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Exploring the effects of dietary restriction and macronutrient composition on life-history traits, in a non-model vertebrate system.

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Abstract

Dietary restriction (DR), is a reduction in food intake, either through overall calorie or specific macronutrient intake, while avoiding malnutrition. DR has been consistently shown to increase longevity and protect against age related diseases. Although originally thought to be the result of a reduction in caloric intake, recent evidence suggests that the ratio of macronutrients, particularly that of protein : non-protein energy, also plays a role. The broad range of species in which DR is known to be effective, suggests an evolutionary conserved mechanism. However, the suggestion of a strong model species bias and a potential sex bias have led some to question the ubiquity of responses to DR. Here, I address the following questions: (i) How consistent is the effect of DR on reproduction? (ii) How does varying macronutrient intake effect both growth and body composition in three-spine sticklebacks (*Gasterosteus aculeatus*)? (iii) What is the effect of dietary macronutrient intake on lifespan and reproduction in the three-spine stickleback? and (iv) How does changing macronutrient intake impact fitness related traits, such as condition and performance in three-spine sticklebacks?

Through use of a systematic review and meta-analysis, I show that the effect of DR on reproduction is evolutionarily conserved, though the effect is stronger in model species. However, when accounting for all significant moderators there is no evidence of the suggested sex differences in the effect of DR. I show that body composition is predicted by dietary lipid intake, with sticklebacks targeting a lower ratio of protein : fat within the body, potentially via metabolism and excretion of protein. These results hint at a link between conversion and excretion of protein and survival costs associated with high protein diets. I show that mortality risk is reduced at balanced protein : lipid intakes in males and generally at low protein : lipid intakes for females. However, the effect in females is not consistent throughout life. I further show that reproduction is maximised on high protein : lipid intakes for both sexes. These results suggest a macronutrient mediated trade-off between lifespan and reproduction in male three-spine sticklebacks. Finally I show a positive effect of lipid intake on male condition (a possible indicator of overall health) hinting at a relationship between lipid intake, adiposity, health and lifespan in male three-spine sticklebacks.

Lay Summary

It is commonly claimed that “you are what you eat”, with food being well known to affect health. Dietary restriction (DR) is a reduction in food intake and has been consistently shown to both improve overall health and increase lifespan. However, it is unclear whether you need to reduce the total amount of food eaten to get the benefit of DR or whether this can be achieved just by reducing particular components. In fact, recent findings suggest that the amount of protein in relation to non-protein components of the diet are key, with calorie intake of less importance. The effect of DR has been demonstrated in a wide range of species, which suggests a consistent pattern and fuels speculation of human applications. However, differences between laboratory and non-laboratory groups as well as differences between the sexes appear to contradict this suggestion. Here using a fish species, the three-spine stickleback, I address the following questions: (i) How consistent is the effect of DR on reproduction? (ii) How does varying nutrient intake affect both growth and body composition in sticklebacks? (iii) What is the effect of nutrient intake on lifespan and reproduction in sticklebacks? and (iv) How does changing nutrient intake affect condition (health) and performance in sticklebacks?

Through a quantitative review of published work, I show that DR consistently reduces reproduction across multiple species, though the effect is stronger in laboratory than non-laboratory groups. Importantly, I did not find any differences between the sexes, which had previously been suggested. I show that the proportion of fat in the body is predicted by the amount of fat consumed, with individuals attempting to reach a balance in the amount of fat and protein in their body. I suggest this is done by expelling excess protein from the body, which hints at a link between removing excess protein from the body and the reduced lifespan typically seen with high protein diets. I show that risk of death was lower on diets which had a balanced protein to fat content for males, but by low protein high fat diets for females. On the other hand, reproduction was greater on high protein low fat diets for both sexes, suggesting that nutrition could play a key role in balancing lifespan and reproduction. Finally I show a beneficial effect of fat intake on a measure of male health, hinting at a link between fat intake, fat storage in the body, health and lifespan in male sticklebacks.

Declaration

The work described in this thesis has been carried out by myself with guidance from my supervisors, unless otherwise stated and detailed below. The thesis is of my own composition and has not been submitted for any other degree or professional qualification.

Chapter 2 JP Moatt carried out data collection, statistical analysis and wrote the manuscript. S Nakagawa and CA Walling assisted in statistical analysis and M Lagisz aided in phylogenetic tree construction. Edits were performed by JPM, CAW, SN and ML.

Chapter 3 data collection was led by JPM with assistance from E Heap, F Moon, and A Kramer. Body composition quantification was carried out by C Hambly and JR Speakman at the University of Aberdeen. JPM performed statistical analysis and wrote the manuscript with guidance and revisions from CAW. D Nussey, C Selman, NB Metcalfe and JRS provided comments on the manuscript.

Chapters 4 and 5 data collection was led by JPM with help from EH, FM, CAW, LJ Mitchell and M Fyfe. CAW and J Hadfield assisted with the statistical analysis. JPM performed the statistical analysis and wrote the manuscript with guidance and revisions from CAW.

Fish diets used in **chapters 3-5** were designed by JPM and CAW with advice from D Peggs and S Davies at the University of Plymouth. Diet manufacture was carried out by DP.

All fish were housed in accordance with home office regulations under a project licence held by CAW (PPL: 60/4310)

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Chapter 1

General Introduction.

1.1 Dietary restriction

Diet has been shown to influence a number of key fitness related traits, such as survival, reproduction and growth (Partridge et al., 2005, Fontana and Partridge, 2015). Dietary Restriction (DR), a reduction food intake without malnutrition resulting in extended lifespan and protection against age related diseases, is one of the most consistent dietary interventions shown to extend lifespan and has dominated the field of ageing research for many years. Although the work of McCay et al. (1935) is widely credited as being the first paper on DR, work on some form of DR had been published as early as 1917. Osborne et al. (1917) showed that by using nutrition to stunt growth, rats (*Rattus norvegicus*) had longer lives and delayed reproduction. McCay et al. (1935) showed that a 40% restriction of food intake significantly extended lifespan in rats (*R. norvegicus*). This led to a wealth of research into the field of DR so that a basic search of "Dietary Restriction" on ISI Web of Science returns well over 15,000 papers (data of access 02/07/2017). Since the original work using rats, DR has been shown to be effective in a wide range of species, including model lab species such as: yeast (*Saccharomyces cerevisiae*; Jiang et al., 2000), nematodes (*Caenorhabditis elegans*; Lakowski and Hekimi, 1998), fruit flies (*Drosophila melanogaster*; Lee et al., 2008) and mice (*Mus musculus*; Simons et al., 2013), as well as non-model species such as: Primates (Colman et al., 2014), arachnids (Austad, 1989) and fish (Inness and Metcalfe, 2008, Terzibasi et al., 2009). The effectiveness of DR in such a wide range of species suggests an evolutionary conserved mechanism, thus leading to speculation that it may be a viable method of lifespan extension for humans (see below). Here, I will give a brief summary of the DR literature and highlight current areas of debate and interest. Particularly, I will

highlight the debate surrounding caloric versus macronutrient intake and the advent of the geometric framework of nutrition, as well as the questions surrounding the universality of DR.

1.2 Calories or macronutrients?

1.2.1 Calories not macronutrients

From its earliest beginnings, DR was thought to act through a retardation of growth (Osborne et al., 1917). Further exploration suggested the effect of DR was due to a reduction in calories (Mccay et al., 1935), leading to the term calorie restriction (CR), a reduction in calorie intake resulting in extended lifespan, becoming synonymous with DR. It has been suggested that there is a linear relationship between increasing restriction of calories and lifespan extension up to a 60% restriction, with restrictions over 60% causing malnutrition and a reduction in lifespan (Speakman and Hambly, 2007). The two most commonly used methods of achieving CR are a limited daily (LD) regime or every other day (EOD) feeding (Anson et al., 2005). LD is the feeding of a restricted ration daily, based on the daily intake of a control group fed *ad libitum*. In EOD feeding, the control group and restricted group are fed the same daily ration, however the restricted group is starved on alternate days (Anson et al., 2005). CR has been successfully implemented in a wide variety of species, from yeast (Jiang et al., 2000), to primates (Colman et al., 2014).

As the interest in DR increased, the role of macronutrient intake in lifespan extension gained attention, due to restrictions in calorie intake often restricting macronutrient intake simultaneously. Initial evidence suggested that responses to DR

were mediated by the ratio of protein : carbohydrate in the diet, rather than caloric intake, with diets containing low protein and high carbohydrate maximising lifespan (Ross, 1959, Ross, 1961, Ross and Bras, 1971). However, further studies repeatedly showed no effect of macronutrient ratio, but a significant effect of caloric restriction on lifespan (e.g. Yu et al., 1985, Iwasaki et al., 1988, Masoro et al., 1989) so that by the 1990s, it was widely accepted that the increase in lifespan under DR was due to calorie intake (reviewed Speakman et al., 2016). This work in DR typically used experimental designs that focused on manipulating one variable at a time, e.g. protein or calories (reviewed Simpson et al., 2017). However it was observed that in some cases multiple nutrients are regulated independently to avoid over or under consuming key macronutrients, for example, overconsuming carbohydrate to obtain sufficient protein (Simpson and Raubenheimer, 2005). These one variable at a time manipulations were poorly designed to study the effects of multiple nutritional parameters simultaneously (see Simpson et al., 2017). Thus, a powerful integrative network, the geometric framework of nutrition (GF), was developed and has led to a resurgence in the suggestion that macronutrients underpin responses to DR.

1.2.2 The geometric framework of nutrition

The GF, is an integrative framework of nutrition pioneered by Stephen J. Simpson and David Raubenheimer, (summarised Simpson and Raubenheimer, 2012). Originally designed for the study of insect nutrition, it is now seeing widespread use in the field of DR and on vertebrate species as well (e.g. mice, Solon-Biet et al., 2014, Solon-Biet et al., 2015). This framework proposes that any diet can be broken down into an n-dimensional nutrient space (Simpson and Raubenheimer, 1995, Simpson et al., 2004, Simpson and Raubenheimer, 2007, Simpson and

Raubenheimer, 2012). A dimension can be any nutritional parameter of interest; for example, this could be a specific macronutrient, such as protein and lipid, or micronutrients such as specific amino acids. In DR research, the focus is usually on the three macronutrients: protein, carbohydrate and lipid (Fig. 1.1), with either two or all three of these representing the nutrient space.

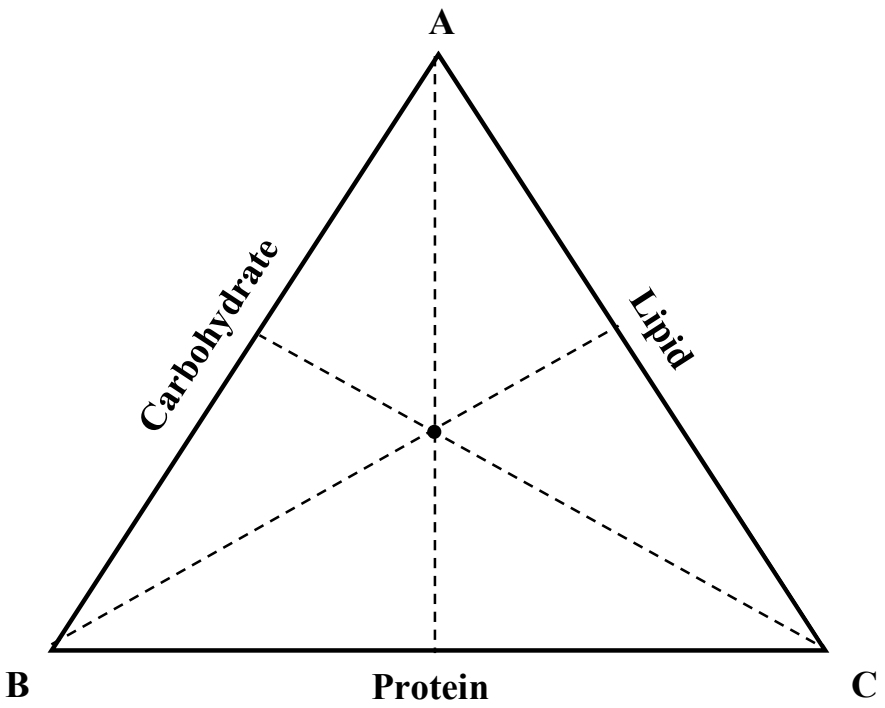

Figure 1.1 Three dimensions of the nutrient space representing the three macronutrients of interest: protein, carbohydrate and lipid. It is these macronutrients that are typically manipulated in DR research. The dashed lines A, B and C represent hypothetical optimal intakes of each specific macronutrient. The intake target, marked by a solid black dot where the lines cross, is where fitness should be maximised. Although here the intake target is presented as a specific point, it is more likely to be a small region of the nutrient space (Figure adapted from Ruohonen et al., 2007).

Life history traits can be easily reconciled with the GF. Any trait of interest, e.g. lifespan, can be plotted as a fitness landscape. In the fitness landscape, specific

macronutrient intakes are plotted against each other with the trait represented as contour lines rising to a peak (Fig. 1.2). The angle of the contour lines indicate which axes is effecting the trait. For example, Fig. 1.2A shows a linear effect of protein intake, with the contour lines running perpendicular to the x axis. Fig. 1.2B shows a linear effect of carbohydrate intake, with the contour lines running perpendicular to the y axis. Fig. 1.2C shows an effect of calorie intake rather than the specific macronutrients, so the contours run diagonally from y to x axis. If there were a non-linear effect, the contours would not be straight lines, but the principles of interpretation remain the same (for examples see: Lee et al., 2008, Maklakov et al., 2008, Fanson et al., 2009, Solon-Biet et al., 2014, Jensen et al., 2015). It is also possible to map the effect of macronutrient intake on a specific trait against a time-varying covariate, such as age, and represent this as a fitness landscape. In this case, the time varying covariate runs along the x-axis and the macronutrient of interest along the y axis. Multiple plots are required, each corresponding to a specific macronutrient of interest (see Maklakov et al., 2009, Jensen et al., 2015 for examples). Fitness landscapes for different traits can then be compared, as they may be maximised on different macronutrient intakes.

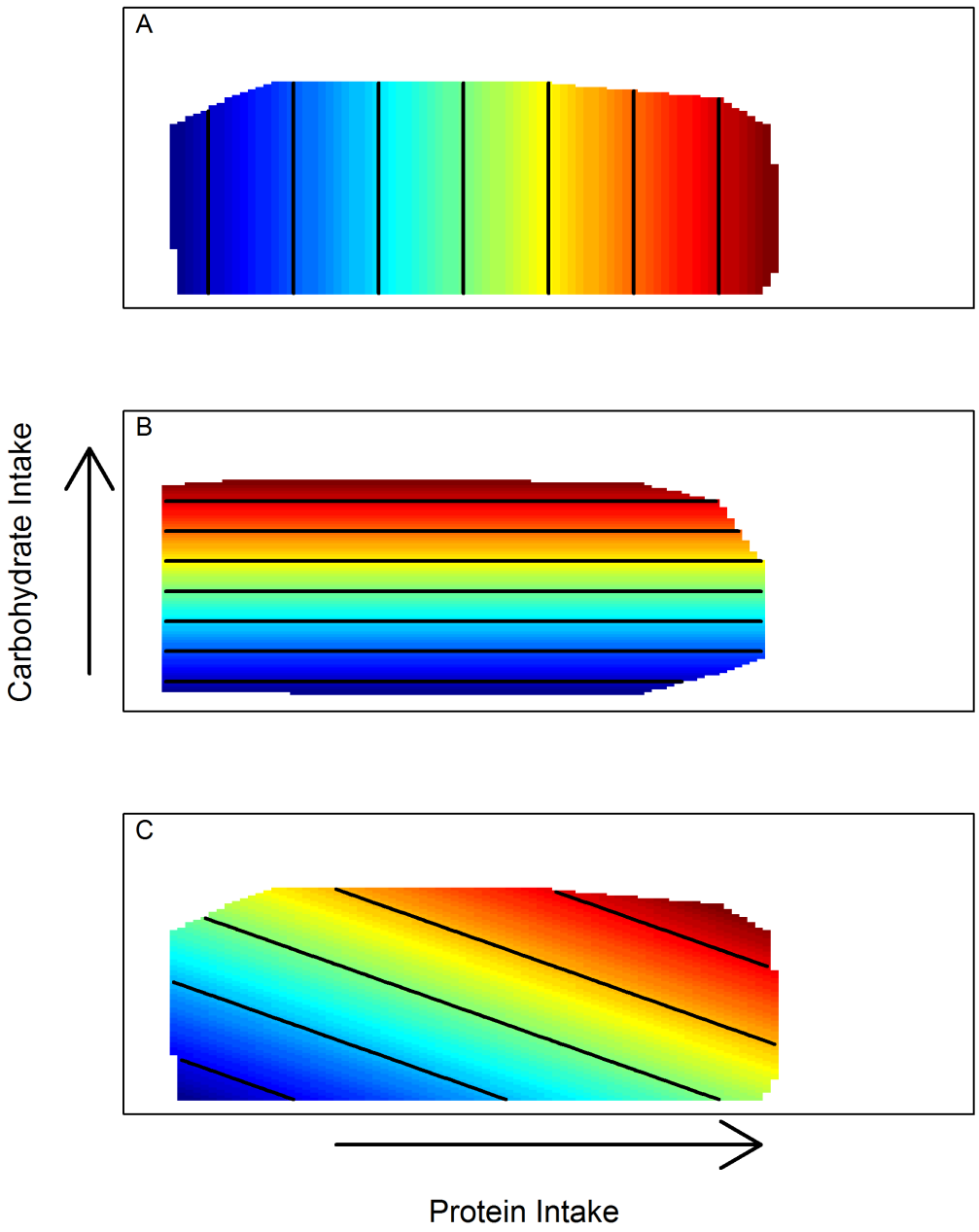

Figure 1.2 Example response surface. Here we can see how a trait of interest can be related to intakes through the GF. The x axis represents increasing protein intake and the y axis represents increasing lipid intake. Contours and colour represent the trait of interest, with colours changing to investment in a particular trait, such as lifespan. Red colours represent high trait value, blue colours represent low trait values. Panels show a trait effected by intake of: protein (A), lipid (B) and calories (C).

The nutrient requirements of any organism will involve optimal levels of intake of each macronutrient and a balance between them. The intake whereby the greatest fitness is achieved has been termed the intake target (Fig. 1.3) and organisms will attempt to get as close to the intake target as possible, to maximise fitness (Simpson and Raubenheimer, 1995, Simpson et al., 2004, Simpson and Raubenheimer, 2007, Simpson and Raubenheimer, 2012). The intake target is not necessarily the same for all members of a species, as age and sex will also play a role (Simpson and Raubenheimer, 1995, Simpson et al., 2004, Simpson and Raubenheimer, 2007, Simpson and Raubenheimer, 2012). It is unlikely that any one food contains the correct balance of nutrients to achieve the intake target (Simpson and Raubenheimer, 1995, Simpson et al., 2004, Simpson and Raubenheimer, 2007, Simpson and Raubenheimer, 2012). Therefore, individuals may need to sample different foods to obtain their intake target (Fig. 1.3).

If an individual is consigned to a single diet, they may be unable to reach their intake target and are therefore forced to compromise ingestion of one macronutrient at the expanse of another. Any compromises will be dictated by the relative importance placed on the different nutrients and the ability of the organism to cope with an excess. For example, over eating carbohydrate to gain sufficient protein may increase lifespan at the expense of reproduction. DR studies employing the GF utilise these compromises to explore the effect of macronutrient intake on fitness traits. To do this, individuals are fed a diet with a specific macronutrient composition, and the effect various traits are measured. By using large numbers of individuals and numerous diets with varying macronutrient compositions, it is possible to identify how macronutrients affect specific traits.

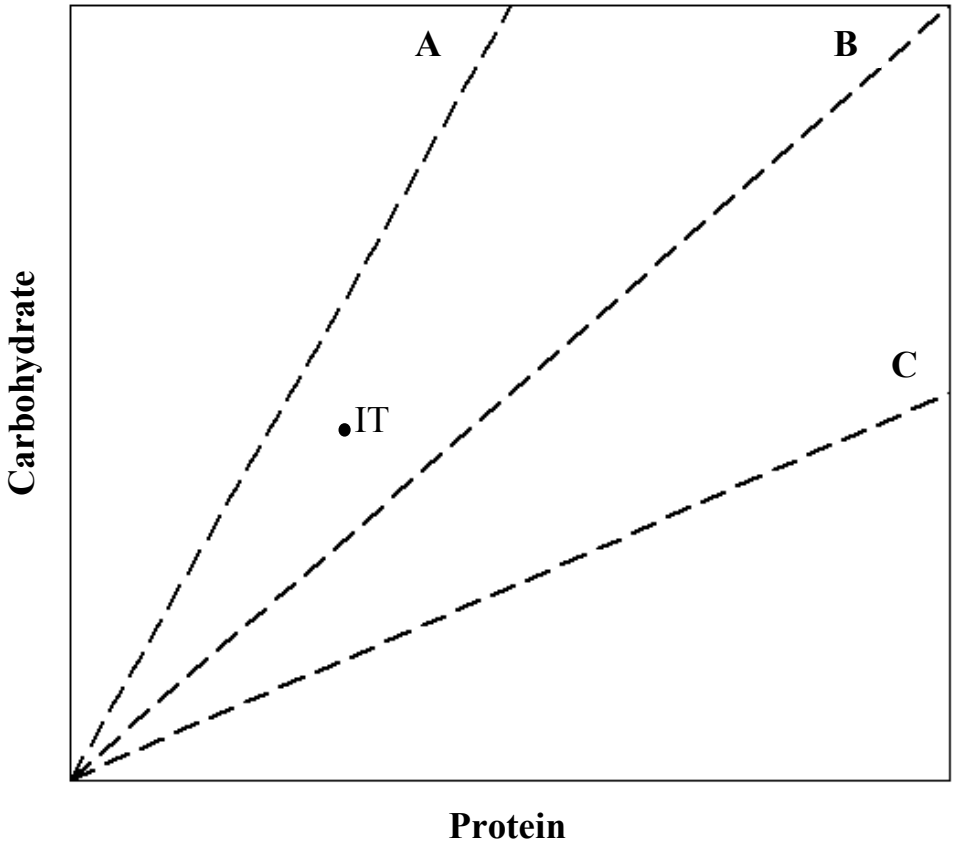

Figure 1.3. Nutrient rails in the geometric framework. The X axis represents protein intake and the Y axis represents carbohydrate intake. The dashed lines (A-C) represent three different foods or diets, with particular ratios of the two macronutrients. IT marks a hypothetical intake target. By selecting a combination of the three diets, an individual could reach the intake target. However, if diet A was not present, the intake target would be unreachable, therefore individuals would consume more of diet B in an attempt to get as close to the intake target as possible and thereby maximise their fitness (figure adapted from Simpson and Raubenheimer, 2012).

1.2.3 Macronutrients not calories.

The development of the GF has led to a much more defined way of performing sophisticated manipulations of diet. This has facilitated a resurgence in work exploring macronutrient balance within the field of DR. By varying a number

of nutritional parameters, the GF allows simultaneous analysis of the effect of caloric intake and macronutrient balance, facilitating identification of the key components of the diet underpinning the DR response. One of the earliest studies to explore DR through the GF was performed by Lee et al. (2008), using *D. melanogaster*. Flies were fed diets with varying protein : carbohydrate ratios and also different caloric densities. Lee et al. (2008) found that: lifespan was maximised on protein : carbohydrate ratio of 1:16, egg laying was maximised on a ratio of 1:2 and overall fitness (measured as lifetime egg production) was maximised on an intermediary intake of 1:4 (Lee et al., 2008). Interestingly, this study found no effect of CR on lifespan, with lifespan declining as the ratio of protein : carbohydrate increased (Lee et al., 2008). This lack of a CR and an effect of protein : carbohydrate ratio on lifespan and reproduction has been repeatedly shown in *D. melanogaster* (Jensen et al., 2015, Lee, 2015) and a similar pattern has been repeatedly shown in a number of insect species including: the field cricket, *Teleogryllus commodus* (Maklakov et al., 2008, Maklakov et al., 2009), the tephritid fruit fly, *Anastrepha ludens* (Carey et al., 2008) and the Queensland fruit fly, *Bactocera tyroni* (Fanson et al., 2009). Furthermore, a similar pattern has been shown in mice, where ratio of protein : non-protein energy was determined to be the key predictor of longevity (Solon-Biet et al., 2014, Solon-Biet et al., 2015). A large meta-analysis of 145 studies revealed that there were quadratic effects of both protein and caloric intake on risk of death, but that the effect of protein was stronger than that of calories (Nakagawa et al., 2012). Therefore, the definition of DR has changed and has ceased to be synonymous with CR. For the remainder of this thesis, DR will be used to describe any restriction in

food intake, encompassing both macronutrient intake and caloric intake. While CR will be used to describe any intervention that only restricted calorie content.

In insect literature, it is widely accepted that the ratio of protein : carbohydrate has a greater effect on lifespan and reproduction than calorie intake alone, however its effectiveness in mammals has been questioned. A series of studies comparing CR and protein restriction in mice, found no effect of protein restriction on a wide number of health related measures (see Mitchell et al., 2015a, Mitchell et al., 2015b, Mitchell et al., 2015c, Mitchell et al., 2016). These studies conflict with the findings of Solon-Biet et al. (2014, 2015), who found that dietary macronutrient content of the diet was driving changes in lifespan and reproduction, rather than caloric intake. Interestingly, throughout the studies of Mitchell et al., protein restriction was not applied using the GF, rather they performed a more classical set up of diets with a specific protein percentage in which they then restricted protein (e.g. a 10% protein restriction). To maintain the caloric intake, as they restricted protein, they increase carbohydrate (e.g. Mitchell et al., 2015a). Thus each restriction level had a unique ratio of protein : carbohydrate and, therefore, represented a unique nutrient rail. This prevents the authors from performing caloric and protein restriction concurrently as is usual in GF studies (for example see Lee et al., 2008).

A further criticism of macronutrient manipulations is presented in a quantitative review of DR mouse literature (Speakman et al., 2016). Here it is suggested that on the whole, CR with protein restriction increases lifespan, however, CR without protein restriction generates the same effect (Speakman et al., 2016). The authors conclude that CR is driving lifespan extension in rodents and cannot be explained by macronutrient manipulation even when examined using the GF

(Speakman et al., 2016). However, throughout their analysis, protein content of the diet is presented as a percentage, with no quantification of actual protein intake. Thus, there is no way to know if there was any compensatory feeding (Simpson and Raubenheimer, 2005, Simpson et al., 2017). Let us assume there is a certain quantity of protein required to be consumed for peak fitness. Two individuals being fed ad lib on diets containing 10% and 20% protein respectively, would still be able to achieve the specific quantity of protein required. The individual on the 10% diet could simply eat more, thereby increasing the amount of protein ingested. Therefore, neither individual is actually under protein restriction.

It is clear, therefore, that there remains a great deal of uncertainty and debate surrounding the specific dietary intervention required to generate the DR response in lifespan and reproduction. In insects, where the majority of this work has been carried out, the response to DR seems conclusively to be the result of dietary macronutrient balance. On the other hand, the evidence in vertebrates, particularly rodents, is far less clear cut, with the only application of the GF to date being in mice (Solon-Biet et al., 2014, Solon-Biet et al., 2015). If we are to uncover whether the responses to DR in vertebrates are due to macronutrient balance, suggesting a conserved DR mechanism, or CR, suggesting more species specific mechanism, the GF needs to be applied to a wider range of vertebrate species.

1.3 Evolutionary mechanism of dietary restriction.

In order to better understand the effect of DR on lifespan and its potential impact on other fitness related traits, it is important to understand why the lifespan response to DR might have evolved. More generally, this involves understanding and applying the theory of ageing to the field of DR. The theory of ageing most

commonly associated with DR is Kirkwood's disposable soma theory (Kirkwood, 1977), which can be viewed as a phenotypic interpretation of antagonistic pleiotropy (Williams, 1957). Antagonistic pleiotropy proposes that with high extrinsic mortality and low survival to old age, genes which are beneficial in early life, but have negative effects later in life, would still be favoured by natural selection (Williams, 1957). The early life benefit of having this gene would outweigh any late life cost, as the high mortality rates make it unlikely that individuals would survive long enough to face any late life costs (Williams, 1957). Under the disposable soma theory, it is suggested that organisms partition resources between three key life processes: somatic maintenance, reproduction and growth (Kirkwood, 1977). In the absence of extrinsic mortality, the optimal strategy is to invest heavily in somatic maintenance. However, when extrinsic mortality is high, organisms should invest heavily in growth and early life reproduction at the expense of somatic maintenance. Therefore, under normal fully fed conditions, there is an optimum level at which organisms should invest in these three traits, favouring early life reproduction (Fig. 1.4A) (Kirkwood, 1977).

However, under DR it is hypothesised in the Shanley-Kirkwood model that there is a shift in the resolution of this trade off (Shanley and Kirkwood, 2000). A reduction in resources suggests a poor or changeable environment, meaning that when resources are limited, the chances of successful reproduction and offspring survival are low. Therefore, organisms should reduce investment in reproduction, investing more heavily in somatic maintenance in the hope of surviving until the nutrient environment improves (Fig. 1.4B). Once more favourable conditions return,

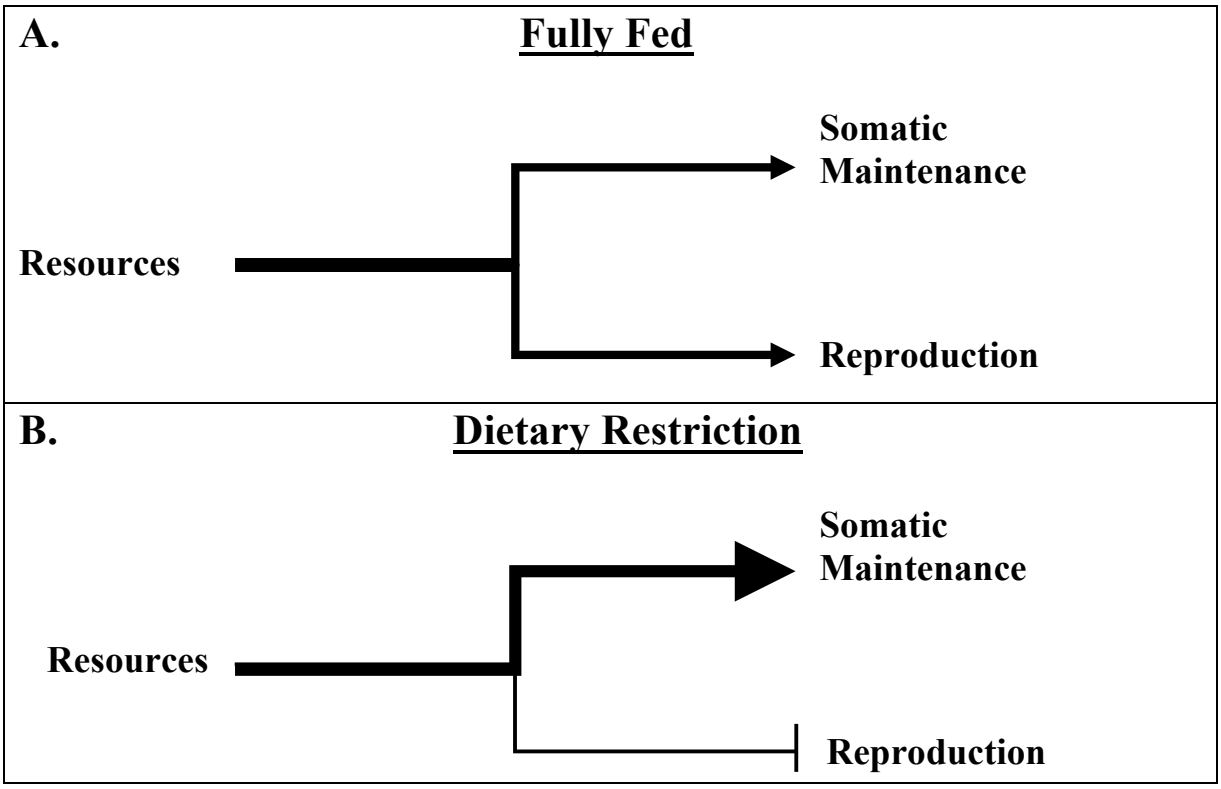

Figure 1.4. Visualisation of the trade-off thought to underpin the DR response. Under fully fed conditions (panel A), resources are partitioned between somatic maintenance and reproduction. However, under DR (panel B), resources are diverted from reproduction and invested in somatic maintenance.

resources can be invested in reproduction once again (Shanley and Kirkwood, 2000). This strategy would only be optimal in changeable environment, i.e. one that has the potential to return to a resource rich environment. If the environment was constantly poor, with no possibility of resources becoming more plentiful, there would be no advantage to postponing reproduction. This initially seems well supported as many studies report a reduction in reproduction and extension of lifespan under DR (e.g. Ball et al., 1947, Chippindale et al., 1993, Chapman and Partridge, 1996).

However, the resource partitioning model described above (Fig.1.4) is not universally accepted. The essential prediction of disposable soma in relation to DR is a reduction in reproduction as lifespan increases (Shanley and Kirkwood, 2000).

However, many studies fail to detect either the reduction in reproduction or the corresponding lifespan extension (Kaitala, 1987, Boggs and Ross, 1993, Inness and Metcalfe, 2008). Furthermore, results in *D. melanogaster*, suggest the correlation between lifespan and reproduction can be uncoupled entirely (Mair et al., 2004). Here, vitellogenisis was prevented in female *D. melanogaster* using the mutant strain ovo^{D1}, or their ovarian activity was impaired through X-irradiation, meaning they could not produce eggs. However, when these flies were subjected to DR, a significant lifespan extension was still seen (Mair et al., 2004). Finally, the Shanley-Kirkwood model (2000), suggests that the response to DR should only be seen over a very narrow range of restrictions. Once restriction drops below this level survival becomes unlikely and, in line with terminal investment theory (Clutton-Brock, 1984), resources should be heavily invested in reproduction (Shanley and Kirkwood, 2000, Mitteldorf, 2001), yet this is not the case (reviewed Mittledorf, 2001).

Most recently Adler and Bonduriansky (2014) proposed an alternative evolutionary explanation of the response to DR, here called the cell signalling hypothesis. This theory argues that the Shanley-Kirkwood resource partitioning model (2000) described above could never be favoured by natural selection, as any postponement of reproduction would result in a massive loss of fitness. Even if resources are limited, the optimal strategy is still to invest in reproduction, as high extrinsic mortality makes the chances of surviving the period of famine low (Adler and Bonduriansky, 2014). They suggest instead the response to DR evolved as a means to minimise the loss of reproduction during periods of low resources. This is achieved by an increase in cell recycling mechanisms such as autophagy and apoptosis, which increases the internal resources of an individual thus making more

resources available for reproduction. However, in the laboratory environment, an increase in internal resources would have the effect of extending lifespan. In the lab, extrinsic mortality is low, meaning the main causes of death are age related disorders, such as cancer. Inhibition of apoptosis is well known to increase tumour development, and thus cancer formation (Evan and Vousden, 2001), whereas an upregulation of autophagy can reduce age related muscle loss (Rubinsztein et al., 2011). Thus, an upregulation of cell recycling mechanisms would have knock on effect of reducing the risk of old age pathologies, thereby extending lifespan (Adler and Bonduriansky, 2014). Adler and Bonduriansky (2014) suggest that the life extending effect of DR will not be reproducible outside of this benign lab environment as individuals in the wild face much higher extrinsic mortality and are less likely to survive to old age. Therefore, any benefits from an upregulation of apoptosis and autophagy will not be seen and no increase in lifespan detected.

The evidence against the Shanley-Kirkwood model of resource partitioning (2000) is far from conclusive. Mair et al. (2004) showed that lifespan extension under DR was seen in females that were incapable of reproduction. However, impairing reproduction through prevention of vitellogenisis and ablating the ovaries does not prevent resources being partitioned for reproduction. If you consider the analogy of a bucket filling from a tap, where reproduction is the bucket and the tap and water are the resources, removing the bucket does not stop the tap from running (Lessells and Colegrave, 2001, Barnes and Partridge, 2003). Merely preventing reproduction, may not stop resources being portioned for reproduction.

The criticisms of resource partitioning proposed by Adler and Bonduriansky (2014) fail to explicitly consider offspring survival. The authors suggest that

postponing reproduction at any time would result in a large loss of fitness (Adler and Bonduriansky, 2014). However, when resources are low, offspring survival is also likely to be low. If few offspring survive, organisms will face a dramatic loss of fitness regardless of how much they invest in reproduction. Therefore, the optimal strategy may be to do as the Shanley-Kirkwood model suggests and divert resources to somatic maintenance (Shanley and Kirkwood, 2000). If reproduction was attempted during the period of low resource availability and few offspring survived, fitness is low. However, if reproduction was postponed, despite the and low chance of surviving the period of famine, fitness would still be higher as any offspring produced in a period of high resource availability would have a much greater chance of survival. Finally, to date the cell signalling theory is only a verbal description, with no theoretical model to support its' claims. For example, we do not have any suggestion of the level of external mortality required to make investment in current reproduction rather than survival the optimal strategy. Thus, the resource partitioning model is still the most widely accepted evolutionary explanation for the response to DR.

1.4 Are responses to DR ubiquitous?

As discussed above, the taxonomic diversity in which DR has been observed, has led to the suggestion of an evolutionary conserved mechanism. However, the effect of DR is not ubiquitous with the suggestion of biases in favour of model laboratory maintained species and females (Nakagawa et al., 2012). Here, I will discuss these biases, why they might be occurring and whether the effect of DR is really universal.

1.4.1 Sex Bias

One caveat often associated with the Shanley-Kirkwood (2000) resource partitioning model is the presence of sex differences in the response to DR. It has been suggested that females face greater reproductive costs, particularly in gamete production, than males (Parker et al., 1972). Thus, under the Shanley-Kirkwood model (2000), females are able to reallocate more resources from reproduction to lifespan and thus achieve a greater lifespan extension. This appears well supported, for example in *D. melanogaster*, lifespan extension under DR is larger for females than for males (Magwere et al., 2004, Partridge et al., 2005). Furthermore, this bias seems to be a general effect across species, with meta-analytic findings suggesting males receive a 20% smaller lifespan extension through DR than females (Nakagawa et al., 2012). However, although it may be true that females face higher reproductive costs on a per gamete basis, males generally face much higher pre-copulatory costs than females, such as courtship and territory defence. For example, in *D. melanogaster* the most costly aspect of reproduction is courtship, not ejaculate production (Cordts and Partridge, 1996). Furthermore, male *D. melanogaster* are able to increase investment in sperm production and mating duration, without a corresponding effect on lifespan (Moatt et al., 2013, Moatt et al., 2014). Thus, the costs of reproduction are likely to be equal for the sexes (Vinogradov, 1998, Bonduriansky et al., 2008), with the majority of male reproductive costs being pre-copulatory behaviours (e.g. courthsip and territory defence), while female costs are incurred through gamete production. Thus, differences in reproductive costs are unlikely to underpin the reported sex differences in the lifespan response to DR.

An alternative explanation for the presence of sex differences is that they are the result of the differing reproductive strategies of the sexes. Generally, males pursue a 'live fast die young' strategy investing more in early life reproduction (Vinogradov, 1998, Bonduriansky et al., 2008). Consequently, any postponement of early life reproduction may not be the optimal strategy for males to adopt. Therefore, under DR males reduce reproduction to a lesser extent than females and, thus, receive a smaller increase in lifespan.

An alternative potential explanation for the presence of sex differences in the response to DR is experimental design. In many DR studies, individuals are kept in isolation and mated infrequently or not at all. For a female, a single or small number of mating events will still represent a significant cost. For example, a female *D. melanogaster* can use stored sperm from a single mating event, to produce upwards of 500 fertilised eggs (Lefevre and Jonsson, 1962). However, for a male, a single mating event in the absence of competition and no territory defence, is likely to represent a much smaller cost. If males are not facing the major costs of reproduction, any lifespan increase as a result of DR is going to be much more difficult to observe. By keeping individuals isolated, we may be artificially creating sex differences in response to DR.

Given the lack of studies performing direct comparisons between the sexes (Burger and Promislow, 2004), combined with the relatively few studies exploring male reproduction under DR (see Chapter 2), definitive conclusions for the cause of this sex bias are difficult to draw.

1.4.2 Model Species Bias

As discussed, the ability of DR to extend life in a diverse range of taxa suggests that DR has an evolutionary conserved mechanism, leading to speculation of potential human applications. However, it has been suggested that although DR has been observed in a wide range of taxa, the majority of these are populations that have been adapted to laboratory conditions, thus the effect of DR may only be apparent in the lab environment (Hayflick, 1998, Miller et al., 2002, Austad and Kristan, 2003). This was formally tested through a meta-analysis, which reported that DR was almost twice as effective at extending life in the five model species (yeast, nematodes, fruit flies, mice and rats) compared to non-model species (Nakagawa et al., 2012). However, the exact cause of this bias is, as yet, unknown.

It has been suggested that this could be the result of unintentional selection and subsequent adaptation within laboratory populations (Harper et al., 2006). Within the lab environment, there is generally high resource availability, which selects for high fecundity but not longevity (Miller et al., 2002, Austad and Kristan, 2003). Therefore, the effect of DR could really be to return individuals to a more 'natural' condition, i.e. one closer to that in the wild. Where reproduction is lower and, in the absence of extrinsic mortality, lifespan longer. If this were the case, DR would have no effect on wild populations.

An alternative suggestion is that we have far better knowledge of the nutrient and environmental requirements of species that are regularly kept in the laboratory than those that are not (Nakagawa et al., 2012). Model species, such as rats and mice, have been maintained in the lab for many generations and consequently have specific

macronutrient guidelines as well as a precise method of achieving *ad libitum* intakes. Thus, we can be confident that a restriction of 10% means that individuals are obtaining 90% of the required nutrition. However, for wild populations that are brought into the lab, precise macronutrient requirements are often not known and *ad lib* intakes may actually involve overeating. In this case, a restriction of 10% may result in the individual obtaining greater than 90% of the required nutrition. Thus, when we compare the lab and wild groups at a 10% restriction, DR appears to be more effective in the lab population.

This bias becomes particularly pertinent when discussing the role of calorie restriction (CR) versus macronutrient manipulation and the geometric framework (GF, see section 1.2). Although CR has been tested in a range of model and non-models species, the GF has thus far only been tested in populations that have been maintained in the laboratory for many generations (e.g. Lee et al., 2008, Maklakov et al., 2008, Carey et al., 2008, Fanson et al., 2009, Jensen et al., 2015, Solon-Biet et al., 2015). Furthermore, the only vertebrate system the GF has been applied to is the mouse (Solon-Biet et al., 2014, Solon-Biet et al., 2015). No study has applied the GF to a wild derived vertebrate population. Therefore, it is difficult to draw conclusions on the role of calorie and macronutrient intake in the effect of DR, particularly in vertebrates, until a wider range of populations are used, particularly ones not adapted to the laboratory environment.

1.5 Other benefits of DR.

1.5.1 Body weight and composition.

As discussed in the opening of this introduction, the origin of DR stems from work on retarding growth (Osborne et al., 1917) and consequently DR is well known to reduce body weight (e.g. Colman et al., 1998). This result is fairly intuitive, a reduction in food intake results in a reduction in body mass. But CR is also known to affect body composition as well (Colman et al., 1998, Selman et al., 2005, Muzumdar et al., 2008, Hempenstall et al., 2010, Mitchell et al., 2015a). For example, in Rhesus macaques, *Macaca mulatta*, a 20-30% reduction in calorie intake resulted in a reduction in adiposity (Colman et al., 1998) and that this 30% restriction group had the highest survival (Colman et al., 2014). It has also been shown that CR reduced visceral fat in rats and resulted in an extension to both mean and maximal lifespan (Muzumdar et al., 2008). Furthermore, surgical removal of visceral fat, also resulted in an increased lifespan (Muzumdar et al., 2008). The suggestion of a link between DR and adiposity, in addition to the well-known detrimental effect of excess adiposity on health and lifespan (Simpson and Raubenheimer, 2009, Piper et al., 2011), has led to the suggestion that a reduction in adiposity is the primary mechanism through which DR acts to extend lifespan (Picard and Guarente, 2005, Muzumdar et al., 2008).

Although the effect of CR on body composition and growth is well known, less is known regarding the effect of macronutrient manipulation on body composition. In *D. melanogaster*, body weight and lipid-free bodyweight increased with increasing protein : carbohydrate ratio of the diet, with carcass lipid content

highest on a dietary protein : carbohydrate ratio of 1:2 (Lee, 2015). Lifespan was maximised on a protein : carbohydrate ratio of 1:4, but flies on the 1:2 ratio had the second highest mean and maximum lifespans, which is counter to the above suggestion of a link between reduced adiposity and increased lifespan under CR (Picard and Guarente, 2005, Muzumdar et al., 2008). On the other hand, additional studies in *D. melanogaster* conflict with the findings of Lee *et* al (2015), with body weight decreasing with increasing protein intake, due to a decline in body fat (Skorupa et al., 2008, Ponton et al., 2015). Mice maintained on diets with a low protein : carbohydrate ratio had increased body fat (Sørensen et al., 2008, Huang et al., 2013, Solon-Biet et al., 2014), but the longest lifespan (Solon-Biet et al., 2014), again questioning the link between reduced adiposity and increased lifespan under DR (Picard and Guarente, 2005, Muzumdar et al., 2008).

There is a large body of research in agriculture and aquaculture exploring the effect of macronutrients on growth and body composition. These studies generally focus on the cost effective production of meat products for human consumption. However, the conclusions have relevance to studies of the relationship between DR, body composition and lifespan. In chickens, when protein is limiting in the diet, individuals will overconsume feed in an attempt to obtain sufficient essential amino acids, which results in higher carcass fat content (Donaldson et al., 1956, Aletor et al., 2000). Similarly, in lambs, increasing dietary protein content, increases protein and reduces fat deposition in the body (Andrews and Ørskov, 1970). In European whitefish (*Coregonus lavaretus*), protein growth and total growth appear to be maximised with a protein intake of approximately 55% and lipid deposition increased with increasing lipid content of the diet (Ruohonen et al., 2003, Ruohonen

et al., 2007). Fish also self-select for a diet composition of 55% protein and 45% non-protein energy (Sanchez-Vazquez et al., 1999, Rubio et al., Ruohonen et al., 2007).

The general trend, therefore, is that body composition is influenced by the ratio of protein : non-protein energy in the diet, with individuals overconsuming carbohydrate to obtain sufficient protein, at the expense of body composition. These results fit well with the Protein Leverage hypothesis (Simpson and Raubenheimer, 2005). It is suggested in the protein leverage hypothesis that individuals eat primarily to obtain a target protein level, with carbohydrate and fat being overconsumed on low protein diets in an attempt to reach this protein level (Simpson and Raubenheimer, 2005, Sørensen et al., 2008, Huang et al., 2013). Thus, for diets with a low protein : non-protein energy content, we would expect to see individuals ingesting more food with the result of increased adiposity and increased lifespan. Although this is true for mice, as described above, this is not the case for *D. melanogaster*. It is clear, therefore, that more work is needed to explore how macronutrient intake effects body composition and in particular fat storage, and the link between body composition and lifespan under DR.

1.5.2 Activity and physical performance.

Similar to adiposity, physical activity and neuromuscular performance, such as endurance tasks, are commonly linked with health and lifespan. For example, it has been shown that rats with access to running wheels live longer than those without when both groups are subjected to DR via calorie restriction (Mccarter, 1998). A large proportion of an individuals energy budget is utilised for physical activity.

Under CR, individuals typically have a biphasic pattern of activity (see Speakman and Mitchell, 2011). In the short term, individuals subjected to DR have higher activity levels and greater endurance than their *ad lib* fed counterparts (Harrison and Archer, 1987, Russell et al., 1987, Weed et al., 1997, Hambly and Speakman, 2005). However, as the duration of DR lengthens, individuals suffer a chronic reduction in activity, eventually dropping below that of *ad lib* fed individuals (Harrison and Archer, 1987, Russell et al., 1987, Weed et al., 1997, Hambly and Speakman, 2005). It has been suggested that an increase in activity in response to short term food shortage would be advantageous in the wild, as it would improve an individual's ability to find new food sources (reviewed Speakman and Mitchell, 2011). However, there is little to no exploration of how shortage of a specific macronutrients, rather than overall calorie deficit, affects activity and endurance.

1.6 Is DR applicable to humans?

DR has dominated the field of ageing research mostly as a result of the suggestion that it is an evolutionarily conserved method for lifespan extension, and thus may be an effective intervention for humans. However, this suggestion is not universally accepted. Using lifespan and caloric intake data from Japan, it was calculated that the best possible mean lifespan for males would be between 78.3-81.9 years on a diet of 1500 kcal per day (Phelan and Rose, 2005). The average standard lifespan for a Japanese male being 76.7-77.5 years, this represents an increase in lifespan of only 0.8 – 5.2 years (Phelan and Rose, 2005). This suggested upper limit assumes a linear response to longevity with caloric restriction, if the model were to assume a more realistic non-linear response, the value is likely to be lower than this (Phelan and Rose, 2005). Alternatively, it was calculated that if a 42 year old male

were to engage in 30% restriction, for 30 years (until the current mean lifespan), they would see a 2.8 year increase in lifespan (Speakman and Hambly, 2007). Furthermore, if the onset of DR was delayed until the male was 52, this increase would be reduced to 6 months (Speakman and Hambly, 2007). If the increase in lifespan were to be this small, it is unlikely that many would choose to pursue DR.

However, perhaps of more relevance to humans, is the ability of DR to extend health-span as well as lifespan. For example, DR has been shown to reduce neurodegeneration, neuromuscular decline and immunosenescence (Ingram et al., 1987, Jolly, 2004, Martin et al., 2006, Shanley et al., 2009, Terzibasi et al., 2009). Furthermore, DR has positive effects in disease models for more specific human age related disorders, such as: cataracts, spontaneous cancer formation, tumour growth, Alzheimer's disease and many more (reviewed Selman, 2014). Evidence for the effectiveness of DR in humans is hard to come by, owing to the difficulty in standardizing dietary intake for control and restricted groups. However, a study comparing 18 individuals undertaking a DR regime to 18 age matched individuals on a normal diet found that DR provided significant protection against atherosclerosis (Fontana et al., 2004). Furthermore, during the Biosphere 2 experiment, participants were inadvertently subjected to a low calorie diet which was associated with a number of health benefits, such as lower cholesterol and lower blood pressure (Walford et al., 1992, Walford et al., 1999). However, the oldest participant from the Biosphere 2 experiment was noted to be severely emaciated and it is suggested that the diet could not have been maintained indefinitely (Le Bourg, 2010). Furthermore, the participants of Biosphere 2 were undertaking daily strenuous physical labour and leading more active lives, which is often associated with health benefits (Walford et

al., 1999). Finally, no baseline measures of these individuals were taking prior to entering biosphere two, so it is impossible to know how much benefit this restriction provided (Walford et al., 1992 1999).

In reality, implementing long-term DR in humans is, perhaps, unrealistic. Especially with evidence that, under DR, the hunger response does not diminish over time (Hambly et al., 2007). Therefore, research is targeting potential DR mimetics, a compound or intervention that will provide the benefits of DR, without needing long-term restriction of food intake. The three most commonly discussed DR mimetics are: rapamycin, metformin and resveratrol (see Dhahbi et al., 2005, Ingram et al., 2006, Selman, 2014 for comprehensive reviews). However, as we do not yet fully understand the mechanisms through which DR acts, an effective intervention for humans is a long way off.

1.7 Physiological mechanism of DR

As discussed in the previous section, the imposition of DR is unlikely to be a realistic intervention for in humans, thus interest is growing in the design of DR mimetic drugs, which recapture the effect of DR without restricting food intake (reviewed Selman, 2014). To facilitate the design of effective mimetic drugs, we must consider the proximate, or physiological mechanism underpinning responses to DR. Current research in this area primarily focuses on the physiological pathways associated with nutrient sensing. Currently there are two pathways of interest, which form a single network (Bjedov and Partridge, 2011): target of rapomycin (TOR) and insulin/insulin like growth factor-1 signalling (IIS; Fontana et al., 2010). When mutations were generated in the TOR pathway in *C. elegens* and *D. melanogaster*,

lifespan could not be further increased through DR (Hansen et al., 2007, Kapahi et al., 2004). More recently, mutations in the IIS pathway of mice have been shown to result in an increase in maximum lifespan (Lorenzini et al., 2013). So far, every eukaryotic genome sequenced has found a TOR gene (Wullschleger et al., 2006), which would fit with the suggestion of an evolutionary consereved mechanism.

Furthermore, the mode of action of the TOR pathway, fits with the proposed evolutionary mechanism of DR discussed above (section 1.3): the trade-off between reproduction and lifespan. Nutrients, and particularly amino acids, stimulate the TOR pathway, which inhibits cell recycling mechanisms, such as autophagy and apoptosis, thus reducing somatic maintenance (Wullschleger et al., 2006, Fontana et al., 2010, Bjedov and Partridge, 2011). The TOR pathway activates the S6 kinase, which promotes protein synthesis and cell proliferation, ultimately increasing growth and reproduction (Fig. 1.5). Meanwhile nutrients also stimulate IIS through the insulin/insulin like growth factor (IGF). IGF activates phosphoinositide-3 kinase (PI3K) and protein kinase B (AKT). AKT also stimulates the S6K, therefore also leading to an increase in growth and reproduction (Wullschleger et al., 2006, Fontana et al., 2010, Bjedov and Partridge, 2011). Under DR, there would be less stimulation of the TOR pathway, thus leading to lower inhibition of autophagy and apoptosis, resulting in an increase of somatic maintenance. There would also be less activation of S6K by both the TOR and IIS pathways resulting in a reduction of growth and reproduction.

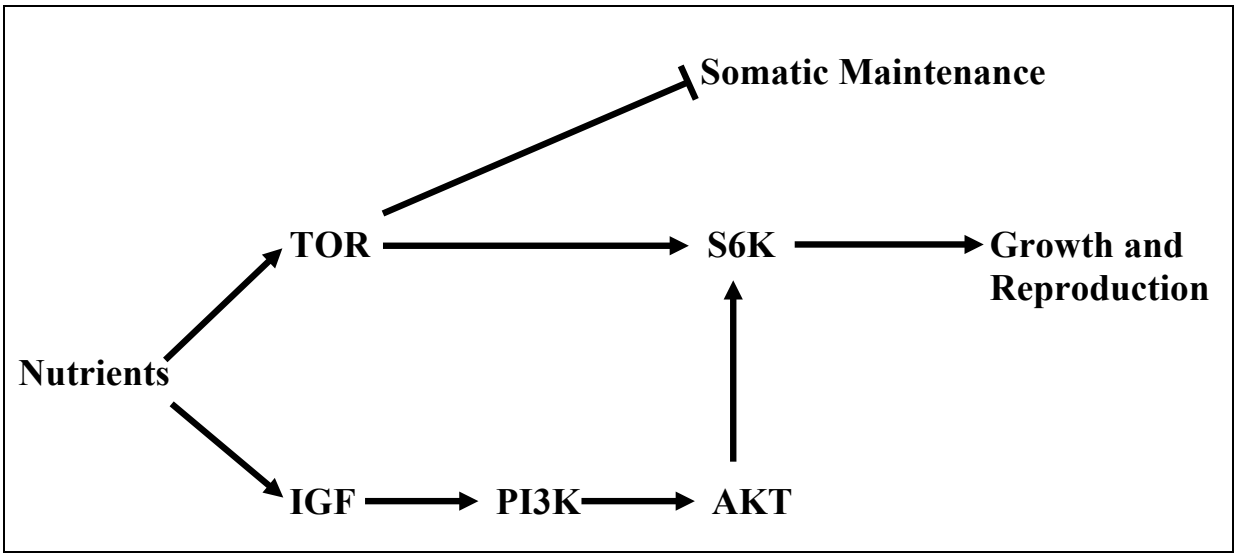

Figure 1.5. Simplified visualization of the TOR and IIS signalling pathways thought to be the physiological mechanism underpinning the response to DR. Nutrients stimulate the TOR pathway which suppresses cell recycling mechanisms such as autophagy and apoptosis (somatic maintenance) and activates the S6 kinases which promotes growth and reproduction. At the same time the nutrients also stimulate the IIS pathway, which also activates S6K, thus promoting growth and reproduction. (Figure adapted from Wullschleger et al., 2006, Fontana et al., 2010, Bjedov and Partridge).

Although DR itself may not be a realistic anti-ageing intervention for humans, the use of a DR mimetic drug to recapture the beneficial effects of DR without food restriction has potential (Selman, 2014). Although the increase in lifespan may be small for humans (Phelan and Rose, 2005, Speakman and Hambly, 2007), a more realistic benefit is in the protection it may provide against age related diseases. Only through understanding the mechanisms underpinning responses to DR, will these mimetic drugs become realities.

1.8 The three-spine stickleback, *Gasterosteus aculeatus*.

It has been suggested that there is a need for a short lived vertebrate species which could serve as a model for ageing research. There is a growing argument that

short lived fish can fill this niche (Gerhard, 2007): with guppies (Gerhard, 2007), zebra fish (Gerhard, 2003) and killifish (Genade et al., 2005) being suggested as viable options. In this thesis, I present experiments using the three-spine stickleback, *Gasterosteus aculeatus*, which also fits the requirements for a useful model vertebrate system (Gerhard, 2007) and has been successfully used for DR studies in the past (Inness and Metcalfe, 2008). Three-spine sticklebacks are short lived teleost fish which are considered part of the Perciformes order (Near et al., 2012). They are found exclusively in the northern hemisphere and typically inhabit coastal or fresh bodies of water. Populations of sticklebacks are abundant but often physically isolated from each other. Sticklebacks can be easily bred in the lab using IVF techniques (Barber and Arnott, 2000) and can be conditioned to feed on pelleted food, thus enabling complex dietary manipulations to be carried out. Sticklebacks have a well-documented breeding cycle which occurs once a year, with both males and females demonstrating high cost reproductive behaviours (Wootton, 1984). For males, these involve the construction of nests, carotenoid based breeding colouration, sperm production and egg fanning (Wootton, 1984). For females, it involves the production of multiple large clutches of eggs (Wootton, 1984). Therefore, by using sticklebacks I am able to assess the effect of macronutrient manipulation in a non-model vertebrate species that has not been maintained in the lab for many generations, and where both sexes can be exposed to costly reproductive behaviours.

1.9 Thesis aims

This thesis will address the following questions:

1.9.1 How universal is the reduction in reproduction under DR?

As discussed above (section 1.3), the evolutionary mechanism suggested to underpin the DR response is the Shanley-Kirkwood resource partitioning model (Shanley and Kirkwood, 2000). However, the evidence in support of this theory is contradictory, with some studies detecting a reduction in reproduction and increase in lifespan under DR (e.g. Chippindale et al., 1993, Chapman and Partridge, 1996), but others failing to see the predicted pattern (e.g. Inness and Metcalfe, 2008). Furthermore, there is some evidence that this trade-off can be completely uncoupled under DR (Mair et al., 2004). Although the generality of the effect of DR on lifespan has been assessed quantitatively (see Nakagawa et al., 2012, Simons et al., 2013), no quantitative assessment of the effect of DR on reproduction, has ever been attempted. Using a systematic review and meta-analytic techniques, I will explore the generality of the effect of DR on reproduction. Specifically, I will focus on four questions: (1) Does DR reduce reproduction? (2) Is there a model species bias? (3) Is there a sex bias? and (4) Does the cost of reproductive trait matter?

1.9.2 Is the effect of DR reproducible in a non-model vertebrate system?

As I have highlighted in section 1.2, there is debate regarding the origin of the DR effect. With classical DR theory suggesting that a restriction of caloric intake triggers this response (e.g. Mccay et al., 1935) and more recent work suggesting a significant effect of macronutrient ratio, particularly the ratio of protein to non-protein energy (e.g. Lee et al., 2008, Maklakov et al., 2008, Carey et al., 2008,

Fanson et al., 2009, Jensen et al., 2015, Solon-Biet et al., 2015). However, as yet this effect has only been tested in insects or mice, all of which have undergone many generations within the laboratory As the effect of DR is well known to be greater in model species (Nakagawa et al., 2012, see section 1.4.2), definitive conclusions are difficult to draw. Therefore, using a wild derived non-model vertebrate, the three-spine stickleback, this thesis will address the following questions: (1) Is lifespan maximised on a low protein : non-protein intakes or is lifespan increased through caloric restriction, when tested in a non-model vertebrate? (2) Is reproduction maximised on a high protein : non-protein intake? and (3) Are there sex differences in the response when both sexes experience a more complete range of reproductive costs (see section 1.4.1)?

1.9.3. What is the effect of DR on other fitness related traits?

How classical CR, effects a range of traits (such as growth, neuromuscular performance, cognitive performance, immune function, etc) has been well studied (see section 1.5 and reviewed Speakman and Mitchell, 2011). However, little research has explored the effect macronutrient manipulation on these traits. If DR is ever to be effectively implemented in humans, we must fully understand all effects that may occur as a result of the manipulation. Therefore, this thesis will also address the following questions: (1) What is the effect of macronutrient manipulation on growth? (2) What is the effect of macronutrient manipulation on body composition? and (3) What is the effect of macronutrient manipulation on neuromuscular performance?

Chapter 2

The effect of dietary restriction on reproduction: a meta-analytic perspective.

As published:

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2.1 Abstract

Dietary restriction (DR), a reduction in food or particular nutrients eaten, is the most consistent environmental manipulation to extend lifespan and protect against age related diseases. Current evolutionary theory explains this effect as a shift in the resolution of the trade-off between lifespan and reproduction. However, recent studies have questioned the role of reproduction in mediating the effect of DR on longevity and no quantitative investigation into the effect of DR on reproduction exists. Here we report a comprehensive comparative meta-analysis of the effect of DR on reproduction. In general, DR reduced reproduction across taxa, but several factors moderated this effect. The effect of DR on reproduction was greater in well-studied model species than non-model species. This mirrors recent results for longevity and, for reproduction, seems to result from a faster rate of decline with decreasing resources in model species. Our results also suggested that not all reproductive traits are affected equally by DR. High and moderate cost reproductive traits suffered a significant reduction with DR, but low cost traits, did not. Although the effect of DR on reproduction was stronger in females than males, this sex difference reduced to near zero when accounting for other co-factors such as the costliness of the reproductive trait. Thus, sex differences in the effect of DR on longevity may be due to a failure to expose males to as complete a range of the costs of reproduction as females. We suggest that future studies should attempt to address the cause of the apparent model species bias and ensure that individuals are exposed to as many of the costs of reproduction as possible. Furthermore, we reveal a general shortage of DR studies that record reproduction, particularly in males, as well as a lack of direct side-by-side comparisons of the effect of DR on males and females.

2.2 Introduction

Dietary restriction (DR), defined as a reduction in food intake without malnutrition (Nakagawa et al., 2012, Jensen et al., 2015), has been shown to extend lifespan and protect against age related diseases across a range of studies (see Nakagawa et al., 2012, Selman, 2014 for current reviews). The majority of studies examining DR use one of five laboratory model species: *Saccharomyces cerevisiae* (Jiang et al., 2000), *Caenorhabditis elegans* (Lakowski and Hemkimi, 1998), *Drosophila melanogaster* (Lee et al., 2008), *Mus musculus* and *Rattus norvegicus* (Simons et al., 2013), hereafter referred to as "model species" (see Nakagawa et al., 2012). The taxonomic diversity of these model species and the fact that the effect of DR is reproducible in other, less commonly studied taxa (e.g. Primates (Colman et al., 2014); arachnids (Austad, 1989); fish (Terzibasi et al., 2009)), has been used to suggest that the effect of DR on longevity is underpinned by an evolutionarily conserved mechanism and may thus have application to humans (Selman, 2014). However, a recent meta-analysis has demonstrated that dietary restriction is nearly twice as effective at extending lifespan in the five model species as it is in non-model species (Nakagawa et al., 2012). Such an overarching pattern questions the taxonomic generality of this effect and thus the suggestion of an evolutionarily conserved mechanism.

The dominant evolutionary explanation of the effect of DR on longevity is based on the disposable soma theory of ageing (Kirkwood, 1997, Shanley and Kirkwood, 2000). Under DR, it is hypothesised that organisms should reallocate resources away from reproduction to somatic maintenance (and thus survival) in order to increase the chance of surviving the period of resource limitation, and thus

reproducing when more favourable conditions return (Shanley and Kirkwood, 2000). A key prediction therefore is that increased longevity is a direct consequence of reduced reproduction. This prediction initially appears well supported; both among and within species fecundity is generally negatively correlated with longevity (Williams, 1966) and many studies cite a negative effect of DR on reproduction. However, close inspection reveals that these citations generally involve one of three studies: two using *D. melanogaster* (Chippendale et al., 1993, Chapman and Partridge, 1996), cited 345 and 362 times respectively, (Google Scholar, accessed 07/09/2016), and the third study using rats (Ball et al., 1947), cited 89 times (Google Scholar, accessed 07/09/2016). More recently, studies have questioned the generality of the longevity-reproduction trade-off underlying the effect of DR, with some data suggesting that longevity and reproduction can be uncoupled (Mair et al., 2004, Leroi, 2001). In *D. melanogaster*, for example, significant lifespan extension through DR was achieved in females that were incapable of vitellogenisis or had impaired ovarian activity and could not produce eggs (Mair et al., 2004). Furthermore, many studies of DR fail to detect a decrease in reproduction, an increase in longevity or both (Kaitala, 1993, Boggs and Ross, 1993, Inness and Metcalfe, 2008). These exceptions and the fact that a small number of studies using model species (where the DR effect on longevity is known to be greater (Nakagawa et al., 2012)) are highly cited to support the longevity-reproduction trade-off underlying DR, suggest that an investigation into the generality of the effect of DR on reproduction is warranted.

One common observation is sexual dimorphism in the response to DR, with lifespan extension greater in females than in males (Burger and Promislow, 2004, Cooper et al., 2004, Magwere et al., 2004). Although direct comparisons between the

sexes within the same study are rare (see below and Burger and Promislow, 2004), the generality of this pattern has been supported by a recent meta-analysis showing a 20% greater lifespan extension under DR in females than males (Nakagawa et al., 2012). An intuitive explanation is that females invest more in reproduction than males. However, although this may be true on a per-gamete basis, males invest heavily in reproduction via other avenues e.g. courtship, intra-male competition and territory defence, such that on average the net costs of reproduction must be equal in males and females (Bonduriansky et al., 2008, Vinogradov, 1998). The fact that male costs of reproduction are generally not associated with gamete production may mean that males have not been exposed to the full costs of reproduction in current DR studies. In many studies males and females are kept separately and often in isolation (e.g. Inness and Metcalfe 2008, Cooper et al., 2004, Carey et al., 2008, Maklakov et al., 2008), and thus males do not experience the costs associated with e.g. courtship and competition. Thus, the sex difference in the effect of DR may be a result of sex differences in the costs of reproduction experienced. If this hypothesis is correct, we would predict a sex difference in the effect of DR on reproductive traits, with DR having more of an effect on higher cost traits. We expect that taking this into account will remove any sex difference in the effect of DR on reproduction.

Another area to explore is how reproductive decline changes with increasing levels of DR. The disposable soma theory of DR predicts an initially linear decrease in reproduction with decreasing resources. However, at very low levels of resources survival becomes unlikely and some degree of terminal investment is predicted (Shanley and Kirkwood, 2000), resulting in a decrease in the rate of reproductive decline. Recently an alternative to the disposable soma theory of DR has proposed

that the response to DR evolved to minimise the loss of reproduction through upregulation of cell recycling mechanisms such as apoptosis and autophagy (Adler and Bonduriansky, 2014). We suggest that this theory also predicts a non-linear reproductive decline with increasing DR. However, in this case the decrease in reproduction should be initially shallow, as cell recycling copes with small reductions in resources via recapture of some internal resources; a faster rate of decline should be observed at higher restriction levels. By examining the pattern of reproduction across levels of DR we can test these two hypotheses.

In this study we therefore attempt to address a number of issues surrounding the effect of DR on reproduction using a systematic review and meta-analysis. This method allows us to combine data from a diverse range of species, across a number of different studies. We can then highlight any general trends in the effect of DR on reproduction, whilst controlling for species-specific and study-specific effects. The specific aims of this paper are thus to investigate: (1) the generality of the effect of DR on reproduction; (2) whether, as for longevity, the effect of DR on reproduction is stronger in model than non-model species; (3) whether, as for longevity, there are sex differences in the effect of DR on reproduction; (4) whether these sex differences can be explained by the likely costliness of the reproductive traits investigated; and (5) the shape of reproductive decline with increasing restriction levels. More generally, this study aims to provide a quantitative summary of the current understanding of the effect of DR on reproduction and thus highlight areas where our knowledge is lacking and further research would be valuable.

2.3 Materials and Methods

2.3.1 Data collection and effect size extraction

Detailed descriptions of data collection and analysis are given in appendix 1 (Appendix 1: Dialog S1.1). Briefly, data were collected through a search of *ISI Web of Science* and *Scopus* using the search strings 'diet* / calor* + restriction + reproduction/fertility/fecundity'. Backward and forward searching was carried out to identify additional papers that were missed in the main database search and the authors' own literature collections on the subject were considered. These searches yielded 1,679 papers (Fig. 2.1), of which 26 reported some measure of reproduction in treated (DR) and control females or males and matched the additional selection criteria (see Appendix 1: Dialog S1.1 for details). This is perhaps a surprisingly low number of studies given the interest in DR and longevity, highlighting the paucity of studies that also collect data on reproduction. Full details for why studies were rejected are provided in Data S3 provided with our data supplement on dryad (doi:10.5061/dryad.3fc02), but a number of studies were rejected as a result of not applying DR consistently across life. It is worth noting that different selection criteria would result in a different selection of studies being included and may affect our results, but we do not think our selection criteria were overly restrictive or would cause any particular bias. The 26 studies used covered 21 species (Fig. 2.2). From these 26 studies we extracted 205 effect sizes (based on 1096 control and 1132 treatment subjects), expressed as Cohen's *d,* calculated as:

$$d = \frac{\overline{x}_1 - \overline{x}_2}{s}$$

where $\overline{x}_1$ represents the mean value of the reproductive measure for the control

group, $\overline{x}_2$ represents the mean for the treatment group and s represents the pooled standard deviation (for s calculation see Appendix 1: Dialog S1.1).

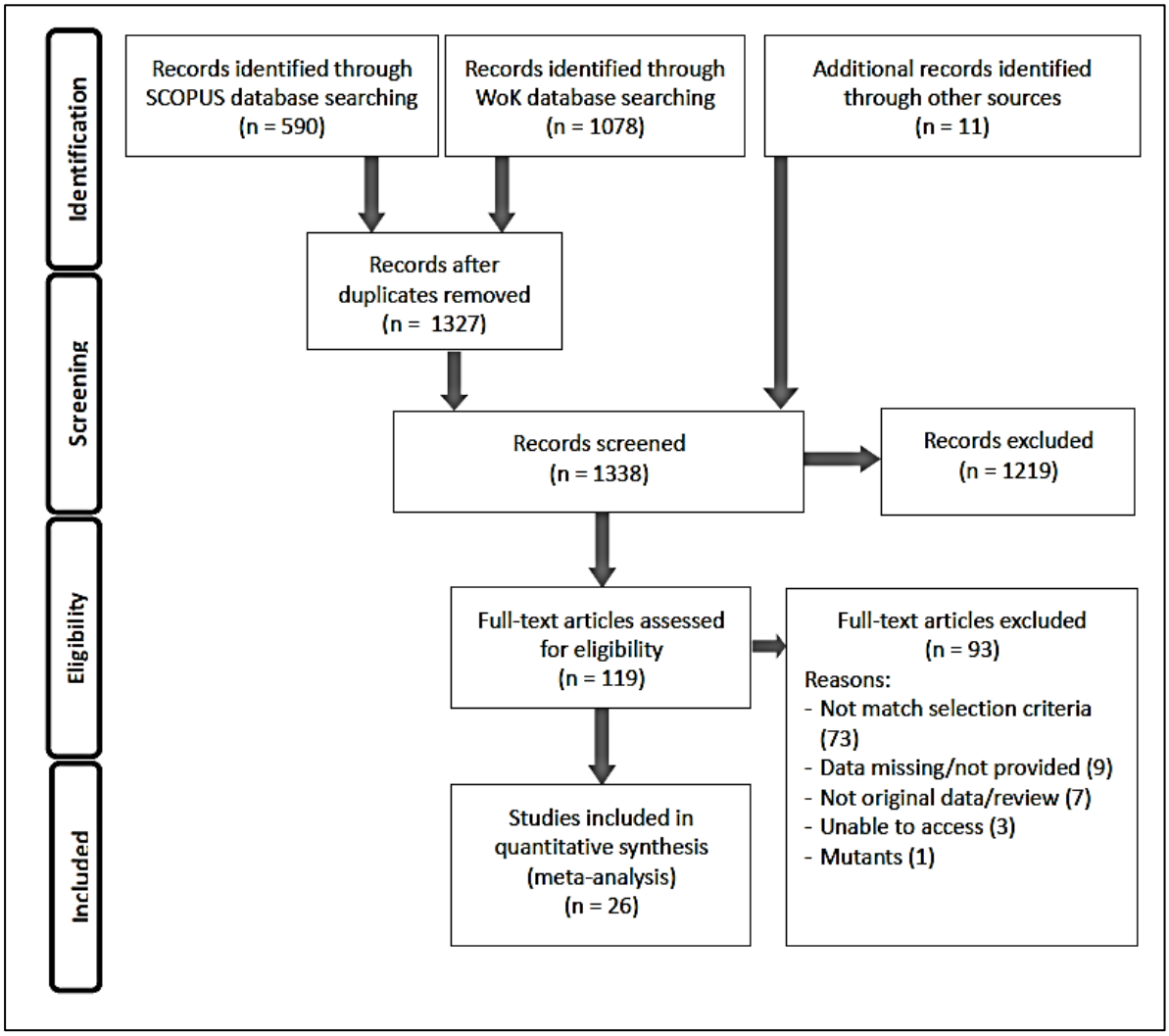

Figure 2.1 PRISMA flow diagram of data collection. The number of papers identified initially through key word searching is shown in the identification boxes. The number of papers excluded is shown for each stage of screening. Reasons for exclusion are given for papers that made it to final eligibility screening.

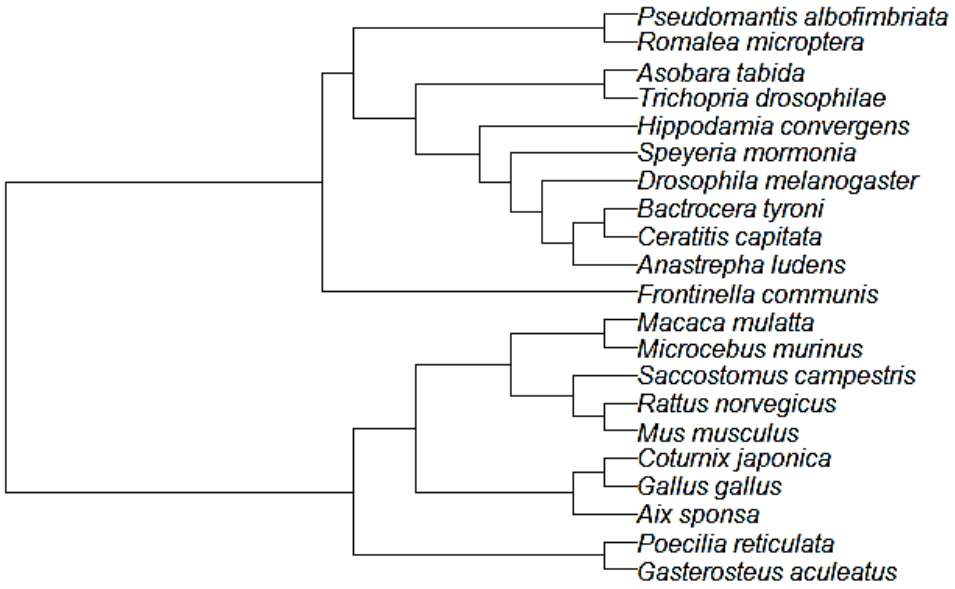

Figure 2.2 Phylogenetic tree of the 21 species used in the meta-analysis. The topological tree (no branch lengths) for all species included in the meta-analysis was produced using the Interactive Tree of Life and polytomies for the insect orders were resolved using Trautwein et al. (2012).

2.3.2 Moderators

In meta-analyses, the use of moderators (e.g. the effect of sex) is often required to explain variation in the effect across studies (heterogeneity (Higgins *et al*. 2003), see Appendix 1: Dialog S1.1). Therefore, we extracted and examined the effect of the following moderators: (1) model species or not, (2) sex, (3) degree of restriction, (4) cost of reproductive trait (see below) and (5) type of control feeding (*Ad libitum* or 100% feeding). As a result of the wide variety of reproductive measures taken, we attempted to categorise reproductive traits based on how much of the total cost of reproduction they were likely to represent. Reproductive traits were

classified as: low cost, moderate cost or high cost (i.e., on an ordinal scale, see Appendix 1: Table S1.1). This measure of cost was graded to take into account species and sex specific costs. For example, in male *D. melanogaster*, ejaculate production was classified as low cost, courtship for a single mating event as medium cost and lifetime courtship investment as high cost. Although subjective, we feel the use of three categories allowed reasonably accurate assignment of traits to a particular category and was necessary to assess how many studies allowed individuals to experience near total reproductive costs. Furthermore, when categorising the cost of trait, we took the study species into consideration, to account for differences in reproductive biology between different species and particularly differences between vertebrate and invertebrate reproductive biology. This also enables cross species comparison, despite the wide variety of reproductive traits being measured.

2.3.3 Statistical analysis

Analysis was carried out in R (R Core Team 2016) using the packages *metaphor* (Viechtbauer, 2010) and *MCMCglmm* (Hadfield, 2010) implementing multi-level meta-analysis (MM) and phylogenetic multi-level meta-analytic models (PMM) (Hadfield and Nakagawa, 2010, Nakagawa and Santos, 2012) (see Appendix 1: Dialog S1.1 for details). We first ran models without moderators to examine overall patterns and to compare phylogenetic and non-phylogenetic models. We then added single moderators to the models to examine their effects in isolation. Finally, we constructed a full model including all moderators of interest. In the results section, we present mean standardized difference between control and restricted groups, standard errors, and 95% credible intervals (CIs). When comparing

phylogenetic models to non-phylogenetic models we present the Akaike information criterion (AIC), which is a model selection index, with the better model having a smaller AIC. Publication bias was examined through visual assessment of the data and through Eggers regression.

2.4 Results and discussion

2.4.1 Does DR reduce reproduction universally?

DR on average resulted in a significant reduction in reproduction (mixed-effect meta-analysis, MM: $\beta_{[meta\text{-}analytic\ mean]} = -0.841$, 95% Confidence Intervals (CI) =[-1.374 to -0.308]). This effect remained robust even when the phylogenetic non-independence of the samples was accounted for (phylogenetic mixed effect meta-analysis, PMM: $\beta_{[meta\text{-}analytic\ mean]} = -0.841$, CI = [-1.374, -0.308], Appendix 1: Table S1.2). However, there was no evidence of a strong phylogenetic signal ($I^2_{[phylogeny]} < 0.001\%$, Appendix 1: Table S1.3) in the effect of DR on reproduction, suggesting a consistent pattern across taxa. Although the model including phylogenetic signal was a better fit by AIC score (phylogenetic AIC = 577.33, non-phylogenetic = 579.86), the improvement was small and was not true for the model including all moderators (see below). To facilitate comparison we present models without phylogenetic signal included from here onwards; results are qualitatively the same for models including phylogenetic signal. Despite the small phylogenetic signal, we observed high heterogeneity amongst studies ($I^2_{[total]} = 98.65\%$, Appendix 1: Table S1.3), suggesting that the reduction in reproduction in response to DR was more apparent in certain studies. As stated above, such large heterogeneity (*sensu* Higgins et al., 2003)

calls for the use of moderators in our models to try to explain variation among studies.

2.4.2 Is there an effect of restriction severity?

As discussed above, an obvious pattern to explore is how reproduction responds to variation in the degree of restriction applied. In general, increasingly severe restrictions appear to increase the lifespan extension achieved by DR, up to the point of malnutrition. However, a linear change in reproduction is not predicted by existing evolutionary theories of DR. We tested these predictions by fitting both a linear and quadratic effect of the degree of restriction. We found a linear negative effect of the degree of restriction (BMM: $\beta_{[Restriction]} = -0.0158$, CI = [-0.0219, -0.0096], Fig. 2.3, Appendix 1: Table S1.4), but no significant quadratic effect (MM: $\beta^2_{[Restriction]} = -0.884$, CI = [-0.925, 2.694], Appendix 1: Table S1.4). This result is intriguing as it is counter to the predictions of both current evolutionary theories of DR (Shanley and Kirkwood, 2000, Adler and Bonduriansky, 2015, Mitteldorf, 2001). One possible explanation for our inability to detect any non-linear pattern is a lack of data at particular restriction levels. Although many of the results analysed here were from studies with reasonably severe dietary restrictions (41 effect sizes, out of 205, with restriction levels greater than 75% of *ad libitum*), there are very few data points with dietary restriction at *very* low or *very* high levels, particularly in model species (Fig. 2.3).

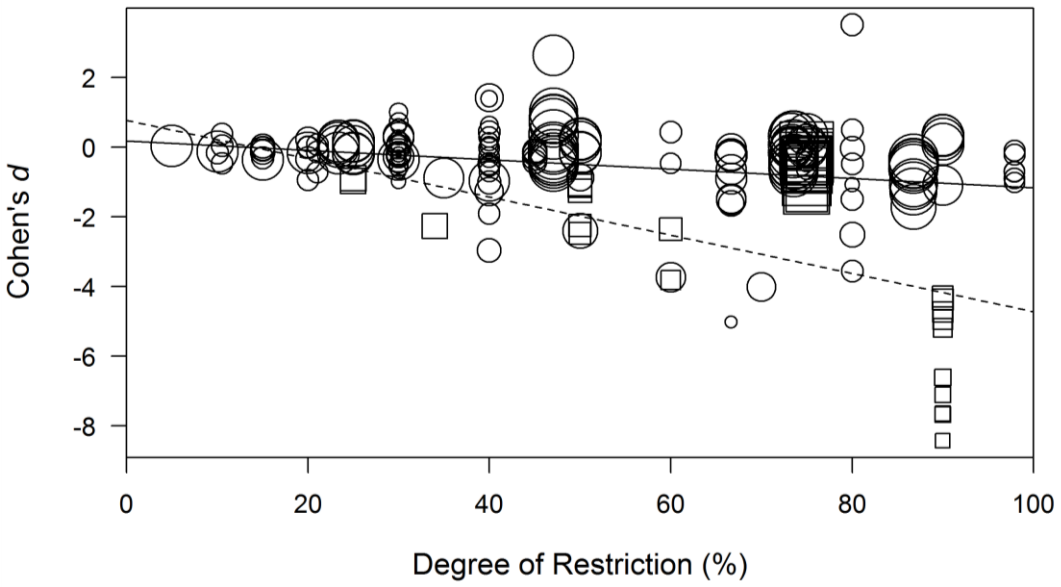

Figure 2.3 The effect of degree of restriction on effect size in model and non-model species. Effect sizes are Cohen's *d*, the standardised mean difference in reproduction between the control and restricted groups (see methods and additional file 1, dialog S1). Model species are represented by squares and the dashed line. Non-model species are represented by circles and solid line. Model species suffer a greater rate of decline in reproduction with increasing degree of restriction. Point sizes indicate the variance in the estimate of the effect size. Details of statistics are given in the main text.

2.4.3 Is there a model species effect?

A recent meta-analysis demonstrated that DR is nearly twice as effective at extending life in model compared to non-model species (Nakagawa et al., 2012). We therefore tested whether such a model species effect was also apparent for reproduction. To allow direct comparison, we defined model species as the same five species used in the meta-analysis on lifespan (Nakagawa et al., 2012; i.e.: *R. norvegicus*, *M. musculus*, *D. melanogaster*, *C. elegans*, *S.cerevisiae*). Our results show that model species suffer a statistically significant reduction in reproduction

(MM: $\beta_{[model]} = -2.42$, CI = [-3.41, -1.43], Fig. 2.4A, Appendix 1: Table S1.5), whereas the reduction in non-model species was lower and marginally non-significant (MM: $\beta_{[non\text{-}model]} = -0.445$, CI = [-0.926, 0.033], Fig 2.4A, Appendix 1: Table S1.5). Comparing these effects, DR had a significantly stronger effect on reproduction in model than non-model organisms (MM: $\beta_{[non\text{-}model/model\ difference]} = -1.97$, CI = [-3.07, -0.87], Fig. 2.4A, Appendix 1: Table S1.5).

In an attempt to disentangle this effect further, we included the interaction between model organism and degree of restriction. This analysis revealed a statistically significant interaction (MM: $\beta_{[restriction * model]} = -0.0415$, CI = [-0.0710, 0.0120], Figs 2.3 & 2.4A, Appendix 1: Table S1.6); the rate of decline of reproduction with increasing DR was steeper in model than non-model species, suggesting that reproduction in model species is more responsive to resource availability than reproduction in non-model species. These results fit well with the findings of Nakagawa *et al.* (2012) and with the disposable soma theory of the effect of DR on longevity, if this increased reduction in reproduction results in more resources being available for reallocation to somatic maintenance. However, the obvious question becomes why do model species have a greater reproductive response to increasing restriction than non-model species?

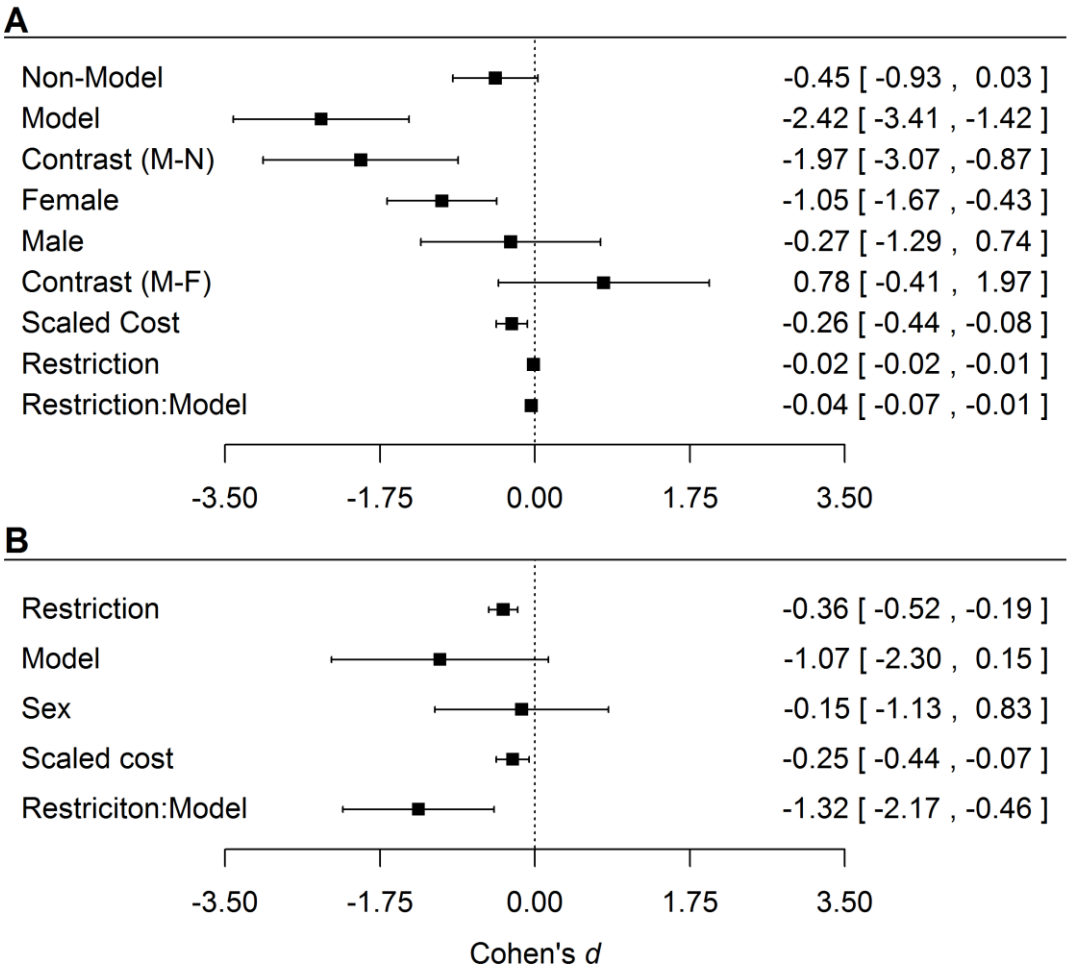

Figure 2.4 Forest plots showing effect sizes (Cohen's *d*, standardised mean difference in reproduction between the control and restricted groups (see methods and additional file 1, dialog S1)) of key moderators for the effect of dietary restriction (DR) on reproduction. Each point represents the Cohen's *d* value with the 95% credible intervals (CIs). Panel (A) represents the outputs from univariate models, with each moderator fitted individually. Each moderator subgroup (e.g. model or non-model species) is represented by a single point. Contrasts represent the difference between effect sizes of the subgroups (e.g. the difference between model (M) and non-model (N) species). Restriction:Model, represents the interaction between degree of restriction (%) and model or non-model species. Panel (B) shows the output from our full model accounting for all moderators, with each point representing the effect size for that moderator.

One possibility is that this is an unintentional effect of selection and subsequent adaptation to the laboratory environment (Harper et al., 2006). For example, the laboratory environment is nutrient rich compared to the natural environment and selects for high fecundity but not longevity (Miller et al., 2002, Austad and Kirstan, 2003). Such an environment may inadvertently favour individuals that have greater plasticity in reproduction in response to nutrient availability. If such plasticity is maintained, either because it has no cost under laboratory conditions or because laboratory conditions vary enough to maintain plasticity, populations that have undergone generations of laboratory selection would be predicted to respond more plastically to food availability than populations that had not undergone such selection. On the other hand, natural environments may be predicted to be more variable than laboratory environments, particularly in food availability, and this may be expected to select for increased plasticity in non-model species. Although a small number of studies compare the effectiveness of DR in extending lifespan in laboratory maintained populations versus wild or wild derived populations (Harper et al., 2006, Miller et al., 2002; Metaxakis and Partridge, 2013), results are inconsistent. It would therefore be interesting to increase the number of these studies and to use a range of food availabilities (rather than just two) to test whether laboratory populations are more plastic to food availability than wild derived populations. If so, inadvertent laboratory selection for high fecundity in a novel environment may have accounted for this plasticity.

Another possible explanation for the increased reproductive response to nutrient restriction in model species is that researchers can more effectively implement restriction in model species (Nakagawa et al., 2012). Model species have

been studied in laboratory environments for many generations and thus diets are more likely to be optimised. In non-model species, where we know less about their nutritional requirements, "*ad libitum*" treatments may actually be fed to excess and foods are unlikely to be optimised. Thus when applying DR, the restricted group may be under a much lower restriction levels than expected in non-model species. For example, a 75% restriction may actually contain 90% of the nutrients needed. Furthermore, the application of the geometric framework of nutrition to DR studies (Simpson and Raubenheimer, 2007, Simpson and Raubenheimer, 2009), has provided a growing body of evidence that specific diet composition affect lifespan and reproduction and that this may be as, or even more, important than classical restriction (e.g. Jensen et al., 2015, Lakawski and Hekimi, 1998, Carey et al., 2008; Maklakov et al., 2008). Studies that use the same species may utilize diets with slightly different composition, which would undoubtedly effect results. It stands to reason, however, that model species which are frequently studied, will have better defined nutrient requirements and therefore that there may be less variation in diet composition and more consistent results. Obviously other explanations are possible, but our results and those of Nakagawa *et al.* (2012) highlight the need for more research to investigate the cause of this model organism effect and how it may affect the generality of the conclusions drawn from investigations of DR.

2.4.4 Is there sexual dimorphism?

We next addressed whether there are sex differences in the reproductive response to DR, similar to those observed in the longevity response (Nakagawa et al., 2012). Our analysis revealed that females suffer a significant reduction in reproduction under DR (MM: $\beta_{[female]} = -1.05$, CI = [-1.67, -0.43], Fig. 2.4A,

Appendix 1: Table S1.7), but that this reduction is much smaller and statistically non-significant in males (MM: $\beta_{[male]}$ = -0.274, CI = -1.291, 0.742, Fig. 2.4A, Appendix 1: Table S1.7). However, when comparing the magnitude of the effect between the sexes, we found no statistically significant difference between males and females (MM: $\beta_{[male / female difference]}$ = 0.776, CI = [-0.414,1.967],Fig. 2.4A, Appendix 1: Table S1.7). The lack of statistical significance in comparison between the sexes is probably because of a lack of statistical power, with the sample size for males being particularly small, only 42 out of 205 effect sizes. These effect size estimates in males come from seven studies, covering five species, all of which were vertebrates (two bird species, one rodent, one primate and one fish species). The remaining studies were on females and there were no studies that allowed side-by-side comparisons of the effect of DR on males and females of the same species. Thus, studies that allow such direct comparison and generally more studies investigating DR in males would be desirable avenues of future research.

2.4.5 Does the cost of the reproductive trait measured matter?

It seems intuitive that traits which are more costly or encompass a greater proportion of total reproductive investment, such as lifetime egg production, will suffer a greater reduction under DR than low cost traits, such as producing a single ejaculate. We therefore included the estimated costliness of the reproductive trait as a moderator. High and moderate cost reproductive traits were statistically significantly reduced under DR (MM L: $\beta_{[high]}$ = -1.12, CI = [-1.71, -0.54]; $\beta_{[moderate]}$ = -1.05, CI = [-1.62, -0.48], Appendix 1: Fig. S1.1 and Table S1.8). In contrast, low cost traits suffered a much smaller and statistically non-significant reduction under DR (MM: $\beta_{[low]}$ = -0.244, CI = [-0.861, 0.374], Appendix 1: Fig. S1.1 and Table S1.8). This

result is unsurprising, but has implications for future DR studies. If, as the disposable soma theory of DR suggests, the effect on longevity is due to a decrease in reproduction, future experiments must allow both control and restricted individuals to experience and express high cost reproductive traits. Otherwise, if individuals are only exposed to a small proportion of the costs of reproduction, the differences between control and restricted individuals are expected to be smaller and more difficult to detect. This may be one explanation for the current sex difference in the effect of DR if females are exposed to more of the costs of reproduction than males (see also below).

This point becomes particularly relevant when examining the current data set in detail. As mentioned above, our search criteria resulted in only 42 effect sizes for males versus 163 for females. Of these 42, only 1 was classed as a high cost reproductive trait (a measure combining all reproductive behaviour into a single score of sexual activity), 18 were moderate cost and the remaining 23 were low cost. The distribution for female traits was: 77 high cost, 69 moderate costs and 17 low cost traits. Given the difference in distribution of the cost categories between males and females ($\chi^2_{2df} = 51.30$, $p < 0.001$), it is unclear if the above sex differences in the reproductive response to DR are real or simply reflect difference in the costs of traits that have tended to be measured in males and females. To test this we fitted a final, 'full' model, to assess the effect of the inclusion of all moderators considered on the estimated effects.

2.4.6 Putting it all together

When accounting for all of the individual moderators and the interaction between model species and the degree of restriction, the degree of restriction, the

cost of the trait and the interaction were all statistically significant predictors of the reduction in reproduction under DR (MM: $\beta_{[Restriction]}$ = -0.357, CI = [-0.520, -0.194]; $\beta_{[cost]}$ = -0.252, CI = [-0.436, -0.067]; $\beta_{[restriction : model]}$ = -1.32, CI = [-2.17, -0.47], Fig 2.4B, Appendix 1: Table S1.9). This model had a conditional R^2 value of 78.8% with random effects explaining 33.2% and fixed effects explaining 45.6% of the variation in effect size between studies (Nakagawa & Schielzeth, 2013). When the interaction between model species and restriction was removed, restriction, model species and cost of trait remained as significant predictors (Appendix 1: Table S1.10).

As with the initial models, we also fitted models that accounted for the phylogenetic non-independence of species, with the non-phylogenetic model being the better fit (including interaction, phylogenetic AIC = 530.08, non-phylogenetic AIC = 528.08 (Appendix 1: Tables S1.9 and S1.11); excluding interaction, phylogenetic AIC = 539.22, non-phylogenetic AIC = 537.22 (Appendix 1: Tables S1.10 and S1.12)). This result suggests that the reduction in reproduction observed under DR is robust and phylogenetically conserved ($I^2_{[phylogeny]} < 0.001\%$ Appendix 1: Table S1.13), but that the rate of reduction is greater in model species compared to non-model species. Furthermore, the reduction in reproduction was greater when examining more costly traits. Of particular interest when fitting the full model was the effect of including the cost of the trait on the sex difference in the effect of DR. When accounting for all other moderators, the difference between males and females was reduced (MM: $\beta_{[male / female difference]}$ = -0.151, CI = [-1.132, 0.830] compared to MM: $\beta_{[male / female difference]}$ = 0.776, CI = [-0.414, 1.967] in the model only containing sex, figure 2.4A and B). This result implies that the supposed sex differences in

response to DR are being driven by experimental design, particularly the costs of reproduction experienced by the sexes.

Essential for all meta-analyses is the assessment of potential publication bias, as interpretation of results of meta-analyses assumes minimal publication bias in the literature (Egger et al., 1997). Visual assessment of our data showed no obvious sign of publication bias (Appendix 1: Fig. S1.2). Furthermore, statistical assessment revealed no significant publication bias in our data set once accounting for heterogeneity (Nakagawa and Santos, 2012) (Eggers regression on the 'meta-analytic' residuals; $\beta_{[intercept]} = 0.0780$, S.E. = 0.0778, $p = 0.317$).

2.5 Conclusions

Our results represent the first formal meta-analysis of the effect of DR on reproduction, an important issue given some studies suggesting the effect of DR on longevity can be achieved independently of reproduction (Mair et al., 2004). Above, we present three main findings that suggest explanations for outstanding issues in this field and avenues for future research. First, DR does lead to a reduction in reproduction but, in line with longevity (Nakagawa et al., 2012), this effect is stronger in model species. We discuss a number of possible explanations for this phenomenon. However, it is clear more studies are needed as any bias in patterns from model species as a result of laboratory adaptation have far reaching consequences for the role of DR studies in understanding and mitigating ageing and its application to humans (Selman, 2014). Second, reproduction declines linearly with increasing DR, at odds with both current evolutionary theories of DR (Shanley and Kirkwood, 2000, Adler and Bonduriansky, 2015, Mitteldorf, 2001). It is possible

that our failure to detect a non-linear response of reproduction to DR was due to a lack of data at certain levels of restriction. More work across a broader range of restriction levels is needed to improve our power to detect non-linear effects and thus assess and compare alternative evolutionary hypotheses on DR effects (Tatar, 2011, Flatt, 2014).

Finally, although our results support a sex difference in the response of reproduction to DR, they suggest this may be due to males and females being exposed to different levels of reproductive costs in the majority of experiments. An alternative explanation is that the longevity-reproduction trade-off can be uncoupled, with diets that maximize longevity not necessarily minimizing reproduction and that this effect can be sex specific (Jensen et al., 2015, Maklakov et al., 2008). Definitive conclusions are difficult to draw because relatively few studies investigate the effect of DR on reproduction in males or allow direct comparison of males and females in the same study using a range of diets (but see Jensen et al., 2015, Maklakov et al., 2008). This is presumably because of the difficulty of designing meaningful measures of male reproductive investment that would encompass the majority of the costs. One potential solution is to measure many male reproductive traits and combine them into an overall score of reproductive investment (Devigili et al., 2013). Even if this is not possible, future DR studies must carefully consider the biology of the study organism and ensure both sexes are exposed to as close to the complete costs of reproduction as possible. For males this will usually include allowing costs such as those incurred while attracting females and direct competition with other males. By doing such experiments, we can start to assess whether sex differences in

the response to DR, both in terms of reproduction and longevity, are a real and interesting sexual dimorphism, or an artefact of experimental design.

Chapter 3

Body macronutrient composition is predicted by lipid and not protein content of the diet.

As Published:

Moatt, J.P., Hambly, C., Heap, E., Kramer, A., Moon., Speakman, J. R. & Walling, C. A. 2017. Body macronutrient composition is predicted by lipid and not protein content of the diet. *Ecology and Evolution***.** 2017:1-10. doi:10.1002/ece3.3529.

3.1 Abstract

Diet is an important determinant of fitness related traits including growth, reproduction and survival. Recent work has suggested that variation in protein : lipid ratio and particularly the amount of protein in the diet is a key nutritional parameter. However, the traits that mediate the link between dietary macronutrient ratio and fitness related traits, such as body composition, are less well understood. Here we investigate the relationship between dietary and body macronutrient composition using a first-generation laboratory population of a freshwater fish, the three-spine stickleback. Carbohydrate is relatively unimportant in the diet of predatory fish, facilitating the exploration of how dietary protein to lipid ratio affects their relative deposition in the body. We find a significant effect of lipid intake, rather than protein, on body protein : lipid ratio. Importantly this was not a result of absorbing macronutrients in relation to their relative abundance in the diet, as the carcass protein : lipid ratios differed from those of the diets, with ratios usually lower in the body than in the diet. This indicates that individuals can moderate their utilisation, or uptake, of ingested macronutrients to reach a target balance within the body. We found no effect of diet on swimming endurance, activity or testes size. However, there was an effect of weight on testes size, with larger males having larger testes. Our results provide evidence for the adjustment of body protein : lipid ratio away from that of the diet. As dietary lipid intake was the key determinant of body composition, we suggest this occurs via metabolism of excess protein, which conflicts with the predictions of the protein leverage hypothesis. These results could imply that the conversion and excretion of protein is one of the causes of the survival costs associated with high protein diets.

3.2 Introduction

Variation in diet is well known to be a critical determinant of fitness related traits such as growth, reproduction and survival (Partridge et al., 2005, Fontana and Partridge, 2015). In particular, dietary restriction (DR), a reduction in the intake of calories or particular macronutrients, has been shown to extend lifespan and protect against age related diseases in the majority of species studied to date (see Speakman and Mitchell, 2011, Nakagawa et al., 2012, Selman, 2014 for recent reviews). It is widely accepted that this lifespan extension can be achieved through a reduction in calorie intake (Mccay et al., 1935, reviewed Speakman and Mitchell, 2011). However, recent research has rejuvenated the suggestion that variation in the ratio of specific macronutrients, and in particular a reduction in the protein content of the diet, is a key component of the relationship between diet and lifespan (Carey et al., 2008, Lee et al., 2008, Maklakov et al., 2008, Fanson et al., 2009, Jensen et al., 2015, Solon-Biet et al., 2014, but see Simpson et al., 2017 and Speakman et al., 2016 for discussion). Despite this interest, the traits that link dietary macronutrient intake and lifespan are not currently known. An obvious starting point is the relationship between dietary macronutrient ratio and body composition, especially given the importance of body composition and particularly fat deposition, in determining health and lifespan (Barzilai et al., 1998, Muzumdar et al., 2008). Here, using a freshwater fish as our model, we investigate the relationship between macronutrient ratio of the diet and body composition, as well as how macronutrient ratio impacts on physical performance and activity, two indicators of health and lifespan.

Calorie restriction is well known to affect body weight (Mccay et al., 1935), but is also suggested to affect body composition, particularly adiposity (Colman et

al., 1998, Picard and Guarente, 2005, Muzumdar et al., Hempenstall et al., 2010, Mitchell et al., 2015a) and relative organ size (Selman et al., 2005, Mitchell et al., 2015a). In fact, it has been suggested that a reduction in adiposity is the primary mechanism through which calorie restriction acts to extend health and lifespan (Barzilai et al., 1998, Picard and Guarente, 2005, Muzumdar et al., 2008). In mice, for example, adipose loss due to calorie restriction occurs in a graded manner, mirroring that of lifespan extension (Mitchell et al., 2015a). However, contradictory evidence suggests that fat loss under calorie restriction provided no benefit or was detrimental to lifespan (Liao et al., 2011, Chiba et al., 2014, Park et al., 2017). Thus, although body composition appears to play a role in mediating the effect of calorie restriction on lifespan the exact nature of this relationship is currently unclear.

Similar to calorie restriction, changes in dietary macronutrient composition result in changes to both body composition and lifespan. For example, it has been shown that mice fed high protein : carbohydrate ratio diets have reduced body fat (Sørensen et al., 2008, Huang et al., 2013, Solon-Biet et al., 2014), but surprisingly not the longest lifespan (Solon-Biet et al., 2014). However, a different study found little to no effect of changing dietary protein : carbohydrate ratio on body fat mass (Mitchell et al., 2015a). In *Drosophila melanogaster*, body weight and lipid-free bodyweight increased with increasing protein : carbohydrate ratio of the diet, with carcass lipid content highest on a dietary protein : carbohydrate ratio of 1:2 (Lee, 2015). These flies had the second highest mean and maximum lifespans, with lifespan maximised on a 1:4 diet. However, additional studies in *D. melanogaster* found that with increasing protein intake, there was a decrease in body weight, due to a decline in body fat (Skorupa et al., 2008, Ponton et al., 2015). Thus, as with calorie

restriction, although dietary macronutrient ratio appears to influence body composition the relationship between diet and body composition and lifespan appear complex.

Improving our understanding of how variation in dietary macronutrient ratio influences body composition may shed light on the causes of the lifespan cost of being fed imbalanced diets. An obvious candidate is that there are metabolic or storage costs of excess nutrients merely being absorbed in relation to their relative abundance in the diet. It is known that the body has a limited capacity for storing excess protein, with surplus nitrogen being excreted as urea (Tarnopolsky et al., 1992, Heaney, 1998, Delimaris, 2013). However, there is a positive relationship between fat intake and fat storage, with ingestion of high fat diets resulting in increased fat storage and obesity and thus potentially the associated negative consequences for health and survival (reviewed Hariri and Thibault, 2010, but see Liao et al., 2011, Chiba et al., 2014, Park et al., 2017). The protein leverage hypothesis suggests that individuals eat primarily to obtain a target protein level, with carbohydrate and fat being overconsumed on low protein diets in an attempt to reach this protein level (Simpson and Raubenheimer, 2005, Sørensen et al., 2008, Huang et al., 2013). This hypothesis leads to the prediction that the protein content of the diet will drive the relationship between diet and body composition (Simpson and Raubenheimer, 2005). Studies from agriculture and aquaculture would seem to support this; when protein is limiting, individuals appear to prioritise protein ingestion and consequently overconsume lipid and carbohydrate, resulting in greater adiposity (Donaldson et al., 1956, Andrews and Ørskov, 1970, Aletor et al., 2000, Ruohonen et al., 2003, Ruohonen et al., 2007). If metabolic or storage costs of excess

nutrients are driving the cost of imbalanced diets, we would expect that the protein : lipid ratio of the carcass would be similar to that of the diet and would have the same rank order of protein : lipid ratios as the diets.

An alternative explanation for the survival cost of imbalanced diets, is that animals have the potential to selectively absorb and or excrete particular nutrients and that the cost of an imbalanced diet is due to the costs of these selective processes (Fanson et al., 2012). Under this scenario, body and diet macronutrient compositions would not be expected to match, but body compositions would be expected to be more similar than diet compositions, as individuals selectively absorb or excrete particular nutrients in attempt to reach a target protein : lipid ratio within the body. If individuals are targeting a specific carcass protein : lipid ratio, then the protein content of the carcass would differ across diets. Furthermore, we would expect to see clustering and a reduction in variability in carcass protein : lipid ratio, as individuals would be trying to achieve a particular protein content in relation to their lipid content.

In addition to body composition, physical activity and performance (e.g. endurance) are commonly linked with health and lifespan and are affected by diet. It has been suggested that an increase in activity in response to short term food shortage would improve an individual's ability to find new food sources, thus explaining the commonly observed biphasic pattern of activity (reviewed Speakman and Mitchell, 2011). However, recent evidence suggests that the effect of calorie restriction differs between different components of activity (Mitchell et al., 2016). Currently, there is little to no exploration of how shortage of a specific macronutrients, rather than overall calorie deficit, affects activity and endurance.

Finally, the effect of diet appears to be sexually dimorphic, with lifespan extension under DR greater in females than males (Nakagawa et al., 2012 but see Speakman et al., 2016). It is thought that this sex difference is a result of a differences between males and females in their investment in reproduction (Shanley and Kirkwood, 2000, Moatt et al., 2016), but work exploring the effect of DR on reproduction in males is often lacking (Moatt et al., 2016). One measure of reproductive investment in males is testes mass, but this is often difficult to study as it would require sacrificing males in studies where lifespan is the key trait of interest. In mice, it has been shown that testes mass is only reduced at high restriction levels, suggesting testes are protected against the effect of DR (Mitchell et al., 2015a). The same study reported a marginal effect of protein restriction on testes mass (Mitchell et al., 2015a), but very few other studies look at the effect of dietary macronutrients on testes mass.

Here we used three-spined sticklebacks (*Gasterosteus aculeatus*) reared on diets that varied in macronutrient ratio to investigate the following questions: 1) what is the effect of macronutrient intake on growth and body composition and is this driven by variation in protein content of the diet; 2) how does macronutrient manipulation affect activity and swimming endurance; 3) are there sex differences in the effect of macronutrient manipulation; and 4) what is the effect of macronutrient manipulation on testes size? We predicted that growth would be highest on the diet with the best balance, containing high levels of both protein and lipid. But we predicted the protein content of the carcass would be higher on high protein diets. Furthermore, we expected carcass fat content to be higher with high lipid intake and low protein intake. For endurance and activity, we predicted that; endurance would

be greater on high protein diets, as protein is important for muscle development; while activity would be higher on low protein diets to allow protein restricted individuals to locate better food sources. Finally, we predicted that testes size would be larger on high protein diets.

3.3 Materials and Methods

3.3.1 Husbandry

Experimental individuals were first generation offspring of wild caught three-spine sticklebacks. Parents were collected in the spring of 2014 form Inverleith Pond, Edinburgh, (55.96N 3.22W). Using standard IVF techniques for this species (Barber and Arnott, 2000), 23 clutches were produced, each with a unique sire and dam. Offspring were fed live *Artemia* until one month of age, after which they were provided live *Artemia* and fry powder (ZM Sytems, ZM-100 Fry Food: protein 55.0%, oil 13.0% and ash 12.0%) until three months of age. From three to four months (the start of dietary manipulations) fish were fed standard grade fish pellet (ZM Systems, medium granular: protein 52.0%, oil 12.0% and ash 10.3%) to condition them to surface feeding on fish pellet. At four months of age, fish were molecularly sexed from fin clips and weighed. Fish were then randomly assigned to one of 5 diet treatments (see below), such that an equal number of males and females were assigned to each diet. A total of 150 fish were used, giving 15 fish per sex per diet.

Fish were housed in plastic tanks (30 x 20 x 20 cm), provisioned with an individual air filter and two artificial weeds. Each tank contained three unrelated individuals of the same sex. Individuals were of a different size to enable individual

identification of the fish without physically marking them (Lee et al., 2013). Clutches were evenly split between the tanks to control for both tank and family effects. Light and temperature regimes were matched to natural levels in Edinburgh at that time of year.

3.3.2 Diet treatments

Unlike for mice and flies, where most work on macronutrient ratio has been carried out, it has been shown that carbohydrate is not a key macronutrient for predatory fish, with much more importance placed on lipid (Ruohonen et al., 2003). Therefore, we created five diets differing in the ratio of protein : lipid (Table 3.1). In these diets, protein and lipid are not strongly negatively correlated (see Appendix 2:Fig. S2.1), to allow us to separate the effect of diet into the independent effects of protein and lipid. To achieve this lack of correlation, we used inert carbohydrate filler, which has been shown to be indigestible in teleosts (Kim and Kaushik, 1992, Guillaume, 2001). Thus, although the diets differ in carbohydrate content (Table 3.1), this was indigestible to the fish. Diets were in pellet form made of different combinations of fish meal and fish oil (Appendix 2: Table S2.1). Diets were manufactured at the Aquaculture and Fish Nutrition Centre (University of Plymouth, Plymouth, U.K.).

In the majority of studies where macronutrients are manipulated, diets are provided *ad libitum* with food available at all times. However, as food degrades rapidly in water, this feeding regime is not suitable for aquatic organisms. We therefore adapted a previous feeding regime that has been used in fish (Terzibasi et al., 2009). Here fish are fed to satiation twice per day, in the morning and in the evening. The amount of food provided for each diet was reassessed monthly, by

feeding fish incrementally until satiated. This amount of food was then provided morning and evening for a month until the next reassessment was made. All tanks of the same diet were fed the maximum amount of pellet consumed by any tank on that diet. Fish were maintained on diet treatments throughout the course of the experiment (106 days).

Table 3.1 Table of the nutrient content of the five diets used in this experiment.

Protein (%)	Lipid (%)	Carbohydrate (%)	Ratio P:L	Calories (MJ/kg)
67.5	6.6	15.8	10.2 : 1	19.3
33.2	3.9	53.1	8.5 : 1	17.5
59.3	13.0	16.1	4.6 : 1	20.2
51.6	20.5	17.8	2.5 : 1	22.2
31.2	19.2	39.7	1.6 : 1	21.5

3.3.3 Growth and condition

From the start of diet treatments until the end of the study, fish were weighed and length measured approximately once a month. However, as growth was roughly linear (see Appendix 2: Fig. S2), we only analysed initial weight, to check for any differences between treatments before the start of the experiment, and final weight, to assess differences in growth between diet treatment. Furthermore, a common measure of assessing overall health of a fish is condition index. Here, we use calculated condition using residuals from an analysis of the length-weight relationship (see Bentley and Schindler, 2013):

$$\text{Condition Index} = \log(Weight) - \log(a) - b\log(Length)$$

With the slope (b) and intercept (a) taken from a model of the log of weight against the log of length for all fish measured in this study (Bentley and Schindler, 2013). A negative value indicates a fish in a poorer than average condition and a positive value suggests a better than average condition.

3.3.4 Swimming endurance

On one occasion between days 79 - 100, each fish was assessed for their swimming endurance ability. We used the same protocol as described in Alvarez and Metcalfe (2005). Briefly, fish were placed in a swim chamber (length 25cm, internal diameter 6cm) submerged in a glass sided tank (59 x 29 x 28cm) filled to a depth of 22cm with room temperature water. Fish were exposed to two currents, generated within the swim chamber, initially a slow current (4cms^{-1}) for 5 minutes, to condition individuals to the swim chamber, after which the speed was increased to 20cms^{-1} and a timer started. At the first cessation of swimming, fish were prompted to return to swimming by a small tap on the chamber. If this failed to elicit swimming, or at the second refusal to swim, the current and timer were stopped. Where individuals continued to swim, the trial was allowed to run for a maximum of 30 minutes (5 minutes acclimatisation and 25 minutes at 20cm^{-s}). Immediately following the trail, the fish was removed to a recovery tank and a 50% water change performed before another trial was initiated. Temperature was recorded every two hours, then converted into a daily average. Swimming endurance was taken as the time an individual was able to remain swimming while exposed to the high speed current and any fish that swam for the full trial was given a score of 25 minutes (23 out of 118

tested). Swimming endurance tests were performed with the observer blind to dietary treatment.

3.3.5 Activity

To assess the effect of diet on levels of activity, activity trials were conducted between days 79-100. Activity trials were carried out in a glass sided tank (45 x 25 x 25cm), containing water to a depth of 8cm following a similar protocol to Bolton et al. (2014). The tank was placed on a light box, surrounded by white walls to prevent disturbance and a video camera mounted above the tank. Each fish was placed in the centre of the tank and given a 60s acclimatisation period, followed by eight minutes monitoring. Fish activity was tracked using Viewer^3 tracking software (http://www.biobserve.com/behavioralresearch/ products/viewer/). Activity was measured as the total time spent moving during the eight minute assessment window. Following the assessment period, the fish was removed and a 100% water change was performed prior to the next trial, thereby ensuring there were no chemical cues remaining in the water which could affect the next trial.

3.3.6 Testes mass

At the end of the experiment (24/02/2015), all males were sacrificed through overdose of tricaine mesylate (MS222) and physical destruction of the brain. They were dried, by blotting with paper towel, then both testes were removed and transferred to a pre-weighed Eppendorf. Owing to the delicate nature of the testes, they were not dried prior to weighing. The Eppendorf was then reweighed on a fine balance (± 0.001g) and testes mass was taken as the difference between the two weights (g). Testes measurements were carried out with the observer blind to dietary treatment.

3.3.7 Body composition

On the 25/02/2015, all female fish were also sacrificed through overdose of MS222 and physical destruction of the brain. Carcasses of both sexes were frozen at -20^{o}C until carcass composition analysis was carried out. Wet and dry mass of carcasses were quantified. Soxhlet extraction was used to quantify the fat mass, fat free mass (protein mass) and the remaining carcass was then ashed to determine the bone and mineral content of the samples. We therefore quantified body composition as protein content (g), lipid content (g), ash content (g) and the ratio of protein : lipid in the carcass. Analysing three measures of body composition (ratio of protein : lipid, protein content and lipid content) allows us to test whether changes in the ratio of macronutrients in the body are driven by variation in protein content, lipid content or both. Body composition was analysed blind of the dietary manipulations.

3.3.8 Statistical analysis

All analysis were carried out in R (v3.3.1; R core team, 2016) using the packages *Lme4* (Bates et al., 2015) and *MCMCglmm* (Hadfield, 2010). Tank and family of origin were included as random effects in all models. The ratio of protein : lipid in the carcass was analysed via linear mixed effects (LME) models with Diet and Sex included as categorical fixed effects. Carcass protein, carcass lipid and carcass ash contents were analysed via LME models, with Diet and Sex included as categorical fixed effects and Carcass Dry Weight included as a continuous covariate to account for differences in size. Protein and lipid content of the diets were not strongly negatively correlated (see Fig. S1), therefore we fitted models to try to separate the effects of dietary protein and lipid. These models included the same fixed and random effects as above, but with dietary Protein and Lipid included as

continuous covariates in place of Diet. Testes mass was analysed via LME with Diet as a categorical fixed effect and wet weight included as continuous variable. LME models for wet and dry weight contained Diet and Sex as categorical fixed effects. To assess the effect of diet on activity, we analysed total time moving using LME models with Diet and Sex as factors and Wet Weight as a covariate. Swimming endurance was analysed via a Markov Chain Monte Carlo generalised linear mixed model (MCMCglmm) using a censored exponential distribution, because this data was exponentially distributed, with a number of fish swimming for the full 20 minutes. To minimize autocorrelation of the model it was run for 1,300,000 iterations and a burnin of 300,000 with 1000 samples stored. Diet, Sex, Wet Weight and Water Temperature were included as fixed effects and tank was included as a random effect.

3.4 Results

3.4.1 Growth

There were no significant differences in initial weight or length between the treatments (LME; weight: $\chi^2 = 2.11$; $p = 0.716$; Fig. S2.2; length: $\chi^2 = 1.33$; $p = 0.857$). However, there was a marginally non-significant difference between the sexes in initial weight (LME; $\chi^2 = 3.38$; $p = 0.066$) and a significant effect of sex on initial length (LME; $\chi^2 = 4.75$; $p = 0.029$), with females being slightly larger than males (mean weight (g) ± s.e.: females 0.43 ± 0.02; males 0.38 ± 0.02; mean length (mm) ± s.e.: females 34.20 ± 0.64; males 32.58 ± 0.58). The marginally non-significant difference in initial weight between the sexes disappeared by the final weighing (LME; $\chi^2 = 0.98$; $p = 0.323$), but remained significant for length at final measuring (LME; $\chi^2 = 4.21$; $p = 0.040$; mean length (mm) ± s.e.: females 44.60 ±

0.64; males 42.96 ± 0.79). There was a significant effect of diet on final weight (LME; $\chi^2 = 18.44$; $p = 0.001$; Fig. 3.1A) and final length (LME; $\chi^2 = 13.43$; $p = 0.009$). Post-hoc analysis revealed fish on the *2.5:1* diet were significantly heavier than those on all other diets (Appendix 2: Table S2.2), but longer only than fish on the *8.1:1* diet (Appendix 2: Table S2.3, Fig. 3.1B). However, there was no difference in weight or length for all other diet comparisons (Fig. 3.1, post hoc analysis Appendix 2: Tables S2.2 and S2.3). Diet also had a significant effect on dry weight (LME; $\chi^2 = 28.26$; $p < 0.001$), with post-hoc analysis again revealing this difference was driven by fish on the *2.5:1* diet being significantly heavier than fish on all other diets (post hoc analysis Appendix 2: Table S2.4). As with wet weight, there was no effect of sex on dry weight of the carcass at the end of the experiment (LME; $\chi^2 = 28.26$; $p = 0.197$).

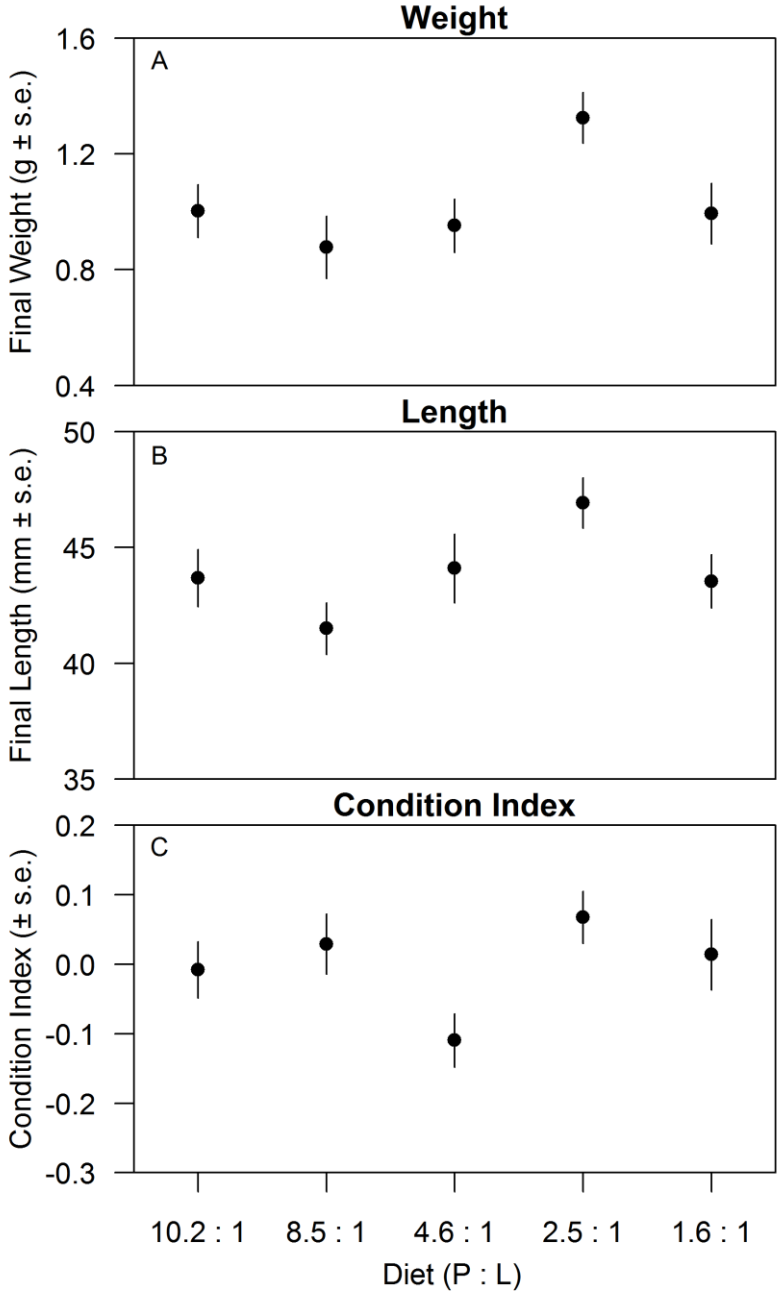

Figure 3.1 The effect of diet (protein : lipid) on (A) Mean final weight (g ± s.e.), (B) Final length of fish (mm ± s.e.) and (C) Mean condition index of fish (± s.e.). There was an effect of diet on final weight (p = 0.001), with individuals on the *2.5:1* diet being significantly heavier than individuals reared on all other diets (all p < 0.040). There was a significant effect of diet on length (p = 0.009), with a significant difference between the 8.*5 : 1* and 2.5 *: 1* diets (p = 0.002). There was a significant effect of diet on condition (p =0.014), with fish on the *4.6 : 1* diet having a lower condition than fish on the *2.5 : 1* (p = 0.009) and *8.5 : 1* (p = 0.045) diets, and a marginally non-significant difference from fish on the *1.6*:*1* diet (p = 0.051).

As with final weight, there was a significant effect of diet on condition index. However, the pattern of differences between treatments for condition index was not the same as that of weight and length. Fish on the *4.6:1* diet were in significantly poorer body condition than fish on the *8.5:1* and *2.5:1* diets, and a poorer but marginally non-significant condition to fish on the *1.6:1* diet (post hoc comparisons Appendix 2: Table S2.5; Fig. 3.1C, Fig. S2.3). There were no significant differences in condition for all remaining comparisons (Appendix 2: Table S2.5). As with final weight, there was no effect of sex on condition index ($p = 0.260$).

3.4.2 Body composition

Analysis of the ratio of protein : lipid in the carcass revealed a significant effect of diet (LME; $\chi^2 = 38.60$; $p < 0.001$; Fig. 3.2; post hoc Appendix 2: Table S2.6). Interestingly, the protein : lipid ratio in the carcass did not match that of the diet, nor show the same rank order. The ratio of protein : lipid was lower in the fish than in the diet that they had consumed, with the biggest difference in fish from the highest protein : lipid diet (Fig. 3.2A). To test this, we analysed the difference between the protein : lipid ratio of the diet and that of the carcass of fish fed on that diet. There was indeed a significant effect of diet. Fish fed on high protein : lipid ratio diets had more of a difference between their body composition and the composition of the diet than fish fed on lower protein : lipid ratio diets (LME; $\chi^2 = 118.59$; $p < 0.001$; post hoc analysis Appendix 2: Table S2.7; Fig. S2.4).

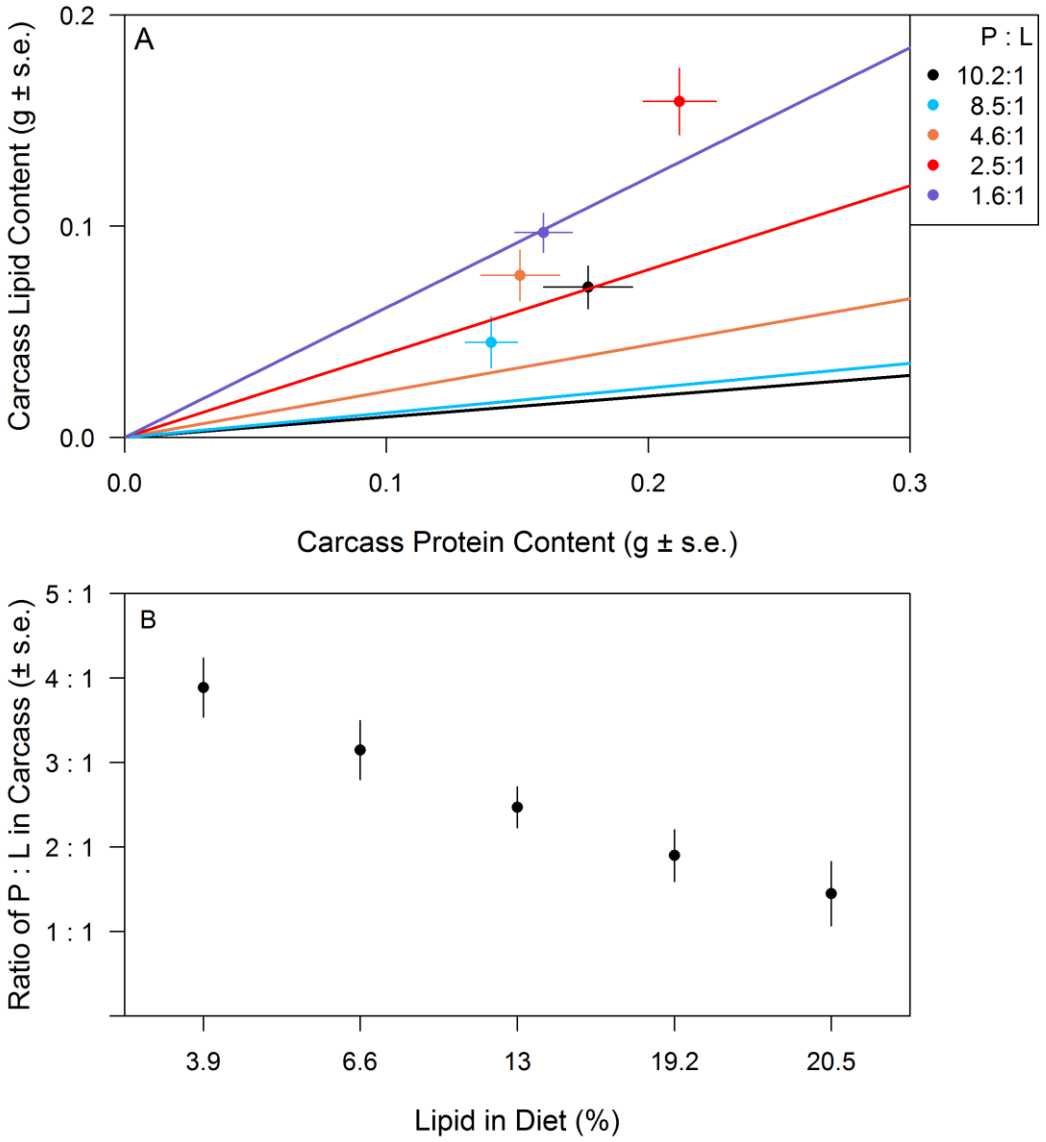

Figure 3.2 (A) Mean (± s.e.) carcass lipid content (g) against mean (± s.e.) carcass protein content (g). Rails represent the protein : lipid ratios in the five diets. Colours correspond to the five diets (see key). There was a significant effect of diet on the degree of difference between carcass and dietary protein : lipid ratio ($p < 0.001$) (B) Mean (± s.e.) carcass protein : lipid ratio in relation to dietary lipid (%). Ratio in carcass is carcass protein (g) / carcass lipid (g). Ratio of protein to lipid in the carcass decreased linearly with increasing dietary lipid intake ($p < 0.001$), but is not significantly affected by protein intake ($p = 0.180$).

Investigating the effect of the protein and lipid content of the diet separately revealed that the carcass protein : lipid ratio was significantly linearly influenced by the percentage of lipid in the diet (LME; $\chi^2 = 37.16$; $p < 0.001$), but not the percentage of protein (LME; $\chi^2 = 1.79$; $p = 0.180$; Appendix 2: Fig. S2.5), with the protein : lipid ratio of the carcass decreasing with increasing lipid content of the diet (Fig. 3.2B). Carcass protein : lipid ratio also differed between the sexes (LME; $\chi^2 = 4.54$; $p = 0.033$), with males having a lower ratio than females (mean ratio of protein : lipid ± s.e.: males 2.3 : 1 ± 0.1, females 2.9 : 1 ± 0.2).

Similar patterns were observed when independently analysing the protein and lipid content of the carcass rather than their ratio. Diet had a significant effect on both protein (LME; $\chi^2 = 53.06$; $p < 0.001$; post hoc analysis Appendix 2: Table S2.8) and lipid content (LME; $\chi^2 = 42.59$; $p < 0.001$; post hoc analysis Appendix 2: Table S2.9) of the carcass when controlling for variation in dry weight (LME: Protein: $\chi^2 = 381.52$; $p < 0.001$. Lipid: $\chi^2 = 261.91$; $p < 0.001$), with protein content of the carcass increasing and lipid content decreasing as the dietary ratio of protein : lipid increased (Fig. 3.3). However, as with carcass protein : lipid ratio, this was driven by a linear effect of dietary lipid content, rather than an effect of dietary protein content: there was a negative linear effect of dietary lipid on carcass protein and a positive effect on carcass lipid (LME; Carcass protein $\chi^2 = 38.23$; $p < 0.001$; Carcass lipid $\chi^2 = 37.50$; $p < 0.001$; respectively; Fig. 3.3), but no effect of dietary protein (LME: Carcass protein $\chi^2 = 0.28$; $p = 0.600$; Carcass lipid $\chi^2 = 0.17$; $p = 0.677$; Fig. 3.3). Finally, there was a significant effect of sex on carcass lipid content (LME; $\chi^2 = 7.76$; $p = 0.005$), with males having greater lipid content of the carcass (mean lipid content (%)

± s.e.: males 28.09 ± 1.10, females 24.72 ± 1.20). However, the effect of sex was marginally non-significant for protein content (LME; $\chi^2 = 3.68$; $p = 0.055$), suggesting that ash content must differ. We therefore analysed ash content, which is a measure of carcass bone and mineral content. There was a significant effect of sex on ash content (LME; $\chi^2 = 5.00$; $p = 0.025$), with females having greater ash than males (mean ash content (%) ± s.e.: males 15.09 ± 0.63, females 16.91 ± 0.63).

3.4.3 Testes mass

There was a positive linear effect of final weight on testes mass (LME; $\chi^2 = 13.17$; $p < 0.001$; estimate ± s.e.:0.00401 ± 0.00111). Accounting for final weight, there was no effect of diet on testes mass (LME; $\chi^2 = 3.96$; $p = 0.412$). However, despite the effect of diet on final weight, there was no evidence of an indirect effect of diet on testes mass, as diet was still non-significant when final weight was excluded from the model (LME; diet: $\chi^2 = 0.864$; $p = 0.930$).

3.4.4 Swimming endurance and activity

The censored exponential model revealed no significant effect of diet, sex, weight or water temperature on swimming endurance (MCMCglmm; all $p > 0.08$; Appendix 2: Table S2.10). To assess activity, we analysed total time spent moving during the eight minute assessment window. This revealed no significant effect of diet, sex or weight on activity level (LME; Diet: $\chi^2 = 3.07$; $p = 0.547$; Sex: $\chi^2 = 0.691$; $p = 0.406$; Weight: $\chi^2 = 0.844$; $p = 0.358$; Appendix 2: Table S2.11).

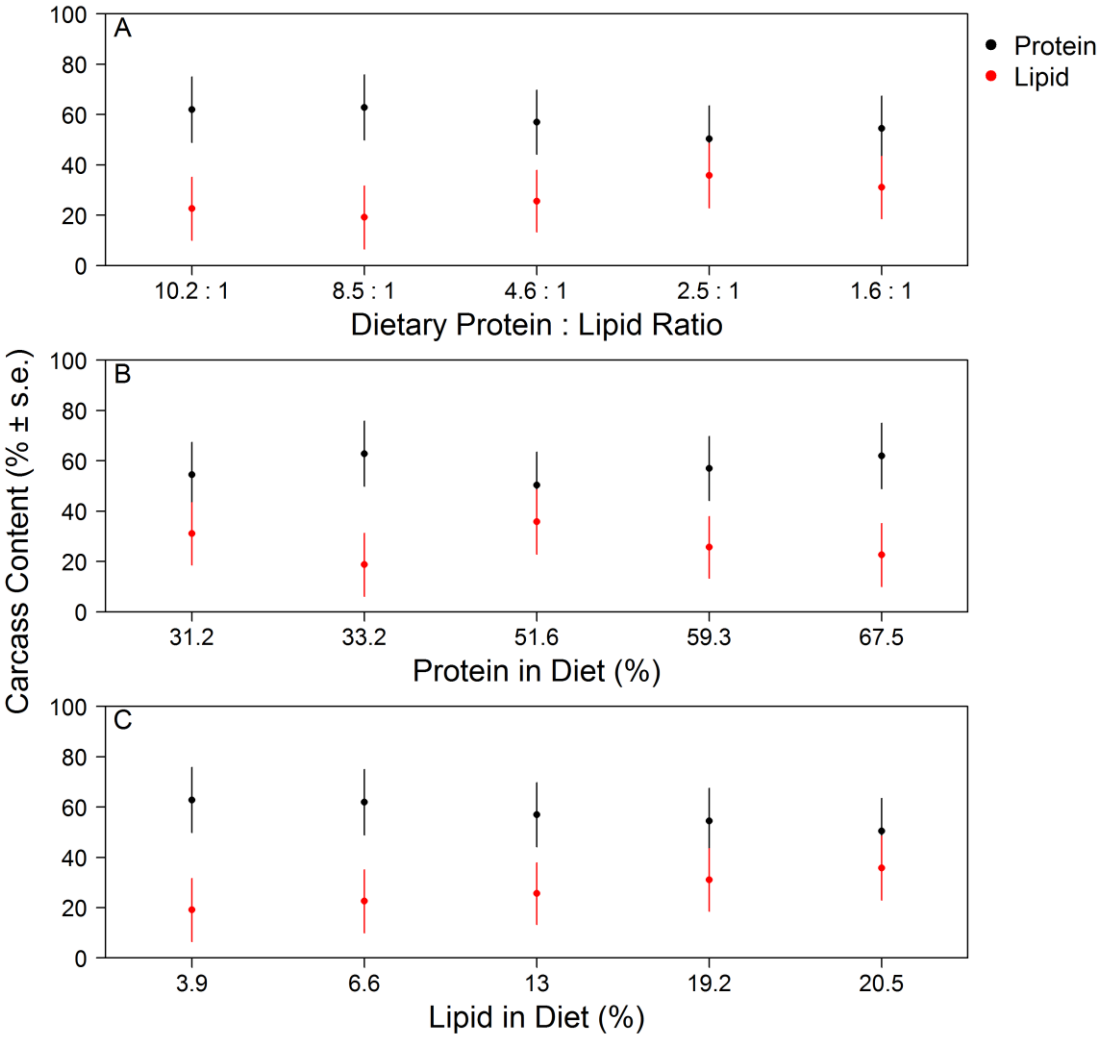

Figure 3.3 Mean (% of carcass ± s.e.) carcass protein (black points) and lipid (red points) content in relation to (A) dietary protein : lipid ratio (B) dietary protein content (%) and (C) dietary lipid content (%). Although there is an effect of diet on both carcass protein and lipid content (both $p < 0.001$), this does not follow the rank order of protein to lipid ratios in the diets (panel A). Carcass protein content decreased and carcass lipid content increased with increasing dietary lipid (both $p << 0.001$; panel C). There was no effect of dietary protein on either carcass lipid or carcass protein content ($p = 0.757$ and 0.648 respectively; panel B).

3.5 Discussion

Diet is known to be an important determinant of key fitness traits (Partridge et al., 2005, Fontana and Partridge, 2015). However, what mediates this effect is much less well understood. Our study explores the relationship between dietary macronutrient ratio and the macronutrient composition of the body, a key determinant of fitness traits such as health. In particular, we explore the direct effect of dietary protein and lipid intake on protein and lipid content in the body. Interestingly, our findings suggest that individuals are able to alter their utilisation or uptake of ingested macronutrients, with the ratio of protein : lipid in the carcass being vastly different from that of the diet. Furthermore, we found no effect of dietary protein intake on body composition, rather carcass protein and lipid content was predicted only by dietary lipid intake. These results would seem to conflict with the predictions of the protein leverage hypothesis (Simpson and Raubenheimer, 2005) as there was no effect of protein intake on body composition and the rank order of protein : lipid ratios were not maintained from the diet to the carcass.

These findings have striking implications for studies exploring the relationship between diet and health or organismal fitness. It has been suggested that being consigned to a specific diet, but fed *ad lib*, allows individuals to increase or decrease their intake of that diet, but prevents them from altering the ratio of macronutrients they ingest (Simpson and Raubenheimer, 1995, Simpson et al., 2004, Simpson and Raubenheimer, 2007). However, our results show individuals clearly alter their utilisation or uptake of the ingested macronutrients, resulting in vastly different macronutrient ratios in the carcass compared to the body. Furthermore, the range of protein : lipid ratios were 1.4:1 to 3.9:1 in the carcasses, but were 1.6:1 to

10.2:1 in the diets. This suggests a pattern of modification towards a lower and less variable carcass protein : lipid ratio. Previous work has suggested that lifespan is maximised on low protein : non-protein intakes, with high protein diets negatively affecting lifespan (Carey et al., 2008, Lee et al., 2008, Maklakov et al., 2008, Fanson et al., 2009, Jensen et al., 2015), which could imply that individuals are targeting lower protein : non-protein ratios in an attempt to increase fitness.

Previous research suggests a survival cost to being maintained on an imbalanced diet. Two obvious alternative explanations for this are the cost of storage of excess nutrients or the cost of their selective absorption or excretion. Our results provide some support for the latter. Individuals fed diets of vastly different macronutrient ratios appeared to converge on more similar body compositions. This suggests that nutrients are not simply stored in proportion to their availability in the diet and thus that survival costs of imbalanced diets are likely associated with selective absorption or excretion of particular nutrients. Given that here, dietary lipid content, not protein, is driving body composition and the positive association between dietary lipid intake and adiposity (Hariri and Thibault, 2010), we suggest that this modification is achieved via metabolism of excess protein. The body has a limited capacity for storing excess protein, which must be converted into urea and excreted (Tarnopolsky et al., 1992, Heaney, 1998, Delimaris, 2013) which may represent one potential cost of a high protein diet (Fanson et al., 2012).

Our results also provide mixed support for the well-known theory of protein sparing in fish, where individuals prioritise lipid use for energy expenditure and use protein for growth and muscle development (De Silva et al., 1991, Vergara et al., 1996, Helland and Grisdale-Helland, 1998). The lack of an effect of protein content

of the diet on protein content of the carcass suggests individual fish were able to maintain the protein content of their carcass on protein intakes as low as 31.2%, and conforms to the theory of protein sparing. However, the negative linear effect of lipid intake on carcass protein content is counter to predictions from protein sparing.

There was little effect of diet on growth, despite diets of differing energy levels being well known to affect size (e.g. Mccay et al., 1935, Colman et al., 1998). However, in our study food was provided *ad libitum*, meaning that despite the diets differing in energy content (Table 3.1) fish on lower energy diets could increase their intake and avoid caloric restriction. Only fish on the *2.5:1* diet were different in size, being significantly larger than all other fish in all other diets. Interestingly, the protein : lipid ratio in this diet, is closest to the ratio that maximises growth in European Whitefish, *Coregonus lavaretus* (Ruohonen et al., 2003). Ruohonen *et al.* (2003), suggested that growth was maximised on a 2.25:1 protein : lipid ratio as this feed had a high energy value. However, this explanation is unlikely here, as food was provided *ad lib* (see above), and there were no differences in growth between other diets differing greatly in energy content (e.g. 17.5 $MJkg^{-}1$ to 21.5 $MJkg^{-1}$). We suggest that the *2.5:1* diet resulted in the greatest growth because it had the highest energy content in combination with a balance of protein and lipid and that high levels of no single dietary component can generate high levels of growth.

Our results also provide evidence of sexual dimorphism in body composition, with males being significantly shorter and having greater fat deposits, and females being longer and having higher bone and mineral deposits (indicated by the higher ash content). These findings fit with a previous study (Kitano et al., 2007), where female *G. aculeatus* were also found to be longer than males. We suggest that this is

likely a result of the different reproductive behaviours exhibited by the sexes. When reproducing, male three-spine sticklebacks defend territories, construct nests, court females and fan eggs, which likely impacts on their ability to forage (Wootton, 1984). Therefore, males potentially invest in fat deposition, rather than growth in length, to provide greater energy reserves prior to the breeding season. This would explain the higher fat content of males here, as our fish were culled immediately prior to the breeding season.

We found no effect of diet on swimming endurance or activity, despite calorie restriction being known to affect activity and endurance (reviewed Speakman and Mitchell, 2011). However, individuals in the current study were fed *ad libitum* and could therefore obtain sufficient energy to maintain activity levels. Additionally, as discussed above, fish appeared able to selectively utilise their ingested macronutrients and therefore may not have been under major macronutrient imbalance, thus there was no stimulation to increase activity levels. Alternatively, these findings could suggest that the effects calorie restriction on performance are not reproducible through macronutrient manipulations. It is also possible that any differences in activity and endurance were too subtle to be detected in the current study.

Finally, we found no direct or indirect effect of diet on testes mass. This could reinforce the suggestion that the testes are protected from the effect of diet (Mitchell et al., 2015a). Alternatively, it could suggest that testes size in the three-spine stickleback is a low cost reproductive trait, and thus that the effect of diet is correspondingly small and therefore difficult to detect (Moatt et al., 2016).

In conclusion, we show that body macronutrient composition differs from that of the diet and that this pattern of variation suggests individuals are attempting to achieve a particular protein : lipid ratio in the body rather than prioritising a single macronutrient. We suggest individuals are achieving a balance of protein and lipid in the body by excreting excess protein. In contrast with a number of recent studies (Skorupa et al., 2008, Sørensen et al., 2008, Huang et al., 2013, Lee, 2015, Solon-Biet et al., 2014, Ponton et al., 2015), our results suggest lipid intake is the key determinant of body composition, rather than protein. Together these data suggest that the key macronutrient for determining body composition may differ between species, which, if this extends to lifespan, has striking implications for studies of DR, where effects have been suggested to be evolutionarily conserved (for example, see Nakagawa et al., 2012, Moatt et al., 2016). Future studies should look to test whether a particular body composition is achieved via protein excretion and whether the costs of excreting protein could be one explanation for the emerging survival cost of being maintained on a high protein diet (Fanson et al., 2012).

Chapter 4

Sex-specific effects of nutrient intake on mortality risk but not reproduction.

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Data collection for this chapter was carried out by JPM, CAW, EH, FM, LJMM, & MF

4.1 Abstract

Dietary restriction (DR) extends lifespan and reduces reproduction in a range of taxa. Originally thought to be due to a reduction in calories, recent evidence suggests that the ratio of protein : non-protein energy is more important. However, this is rarely tested in vertebrates and those studies that do exist have been criticised for reducing calorie intake via dietary dilution rather than restriction. Here, we investigate the role of dietary macronutrient versus calorie content in determining lifespan and reproduction in a vertebrate (the three-spine stickleback) by restricting rather than diluting diets. We find that macronutrient ratio rather than calorie content is more important in determining both mortality and reproduction and that there are sex differences in the effect of macronutrient intake on mortality risk. Male mortality risk was lowest on more balanced protein : lipid intakes and female risk was generally reduced by low protein : lipid intakes, although this effect varied across ontogeny. We did not detect any sex differences in the effect of macronutrient intake on reproduction, or reproductive senescence, with high protein : lipid intakes maximising reproduction in both sexes. This suggests diet may mediate the trade-off between survival and reproduction in this species. Our results provide the first evidence that macronutrients, not caloric intake, predict changes in mortality and reproduction in the absence of dietary dilution. This questions the suggestion of fundamental differences in the mode of action of DR between vertebrates and invertebrates and adds to studies questioning the role of caloric restriction in extending lifespan.

4.2 Introduction

Diet is well known to influence key fitness related traits such as survival and reproduction (Partridge et al., 2005, Fontana and Partridge, 2015). In particular, dietary restriction (DR), a reduction in the intake of calories or specific macronutrients, extends lifespan and protects against age related diseases (see Speakman and Mitchell, 2011, Nakagawa et al., 2012, Selman, 2014 for recent reviews). Originally thought to act through a reduction in caloric intake (calorie restriction), there is a growing body of evidence suggesting that dietary composition, particularly the ratio of protein : non-protein constituents, drives the effect of DR (reviewed Simpson et al., 2017). However, the majority of this evidence comes from studies of invertebrates. Vertebrate studies, particularly in mice show inconsistent results, (e.g. Solon-Biet et al., 2014, Mitchell et al., 2015a) leading to the suggestion that there is a different mode of action of DR between vertebrate and invertebrate species (Speakman et al., 2016). However, comparisons between vertebrate studies are difficult owing to key methodological differences in the implementation of restriction, e.g. dietary dilution versus restriction, which have been suggested to confound results (see Speakman et al., 2016). Here, using a novel vertebrate model, we provide the first test of the effect of macronutrient versus calorie intake on survival and reproduction that avoids the potentially confounding effect of dietary dilution.

DR was originally thought to act through a reduction in calories (Mccay et al., 1935), but recently attention has shifted to the ratio of macronutrients in the diet (reviewed Simpson et al., 2017). This has been facilitated by the application of the geometric framework (GF) of nutrition (summarised Simpson and Raubenheimer,

2012) to DR research (reviewed Simpson et al., 2017). The GF treats the diet as an n-dimensional space where n is the number of dietary components manipulated and any trait of interest can be plotted in this space to visualise the effect of multiple dietary components. By using a large number of diets that vary in both macronutrient ratio and energy content, the effect of calories and macronutrients can be separated. A number of such studies now exist in insects, with a general pattern emerging that macronutrient ratio is more important than calorie content in determining survival, reproduction, and the trade-off between the two, and that survival is maximised on low protein : non-protein energy diets (Lee et al., 2008, Maklakov et al., 2008, Maklakov et al., 2009, Carey et al., 2008, Fanson et al., 2009, Fanson et al., 2012).

However, the ubiquity of the effect of macronutrients on lifespan and reproduction in vertebrates has recently been challenged (see Speakman et al., 2016). Although there is broad agreement that a reduction in protein intake can contribute to some of the effects of caloric restriction, debate is rife over the magnitude of this contribution (discussed Speakman et al., 2016, Simpson et al., 2017). For example, in a rare application of the GF to a vertebrate species, it has recently been demonstrated that the dietary ratio of protein : non-protein energy rather than caloric restriction extends life in mice (Solon-Biet et al., 2014). However, in another series of studies that varied dietary protein content but did not use the GF, Mitchell et al found that protein restriction could not produce the same effects as caloric restriction (e.g. Mitchell et al., 2015a, Mitchell et al., 2015b, Mitchell et al. 2015c). The disparity between studies has been suggested to be a result of important methodological differences (Speakman et al., 2016). Studies utilising the GF and detecting an effect of protein, tend to alter calorie content by diluting the diet rather than restricting the

amount of diet eaten, which may alter the effect of calorie restriction (Speakman et al., 2016). An additional problem is that studies that find an effect of calories but less of an effect of protein tend not to use the GF and thus tend to use fewer diets, reducing the ability to distinguish the effect of calories from one of macronutrients (e.g. Mitchell et al, 2015a). Thus, debate still rages over the role of protein intake in the link between diet, health and lifespan, particularly in vertebrates, and few studies have utilised the GF to separate macronutrient and caloric variation whilst simultaneously restricting rather than diluting the diet.

Another important result in DR studies is the demonstration of sex differences. The effect of DR is stronger in females than in males (Burger and Promislow, 2004, Cooper et al., 2004, Magwere et al., 2004), and this pattern has meta-analytic support, with a 20% lower lifespan extension in males (Nakagawa et al., 2012). The current explanation for this sex difference is that males face lower reproductive costs than females. Thus, given the lifespan reproduction trade-off thought to underpin the lifespan response to DR (Shanley and Kirkwood, 2000), males have fewer resources to reallocate to somatic maintenance under DR. However, direct comparisons of the sexes in the same study are rare (Burger and Promislow, 2004). Furthermore, evolutionary theory questions the assumption of sexual dimorphism in the costs of reproduction. Females face higher costs of gamete production, but males often face greater costs through reproductive behaviour, e.g. competition and courtship (Cordts and Partridge, 1996). Many DR studies fail to expose males to these high cost reproductive activities, thereby making any changes in lifespan more difficult to identify (see Moatt et al., 2016 and Chapter 2). In addition, recent studies separating the role of calories and macronutrients suggest

similarity between the sexes in the effect of diet on lifespan, but sex differences in the effect of diet on reproduction and the trade-off between the two (Maklakov et al., 2008, Jensen et al., 2015). Thus it is clear additional studies are required to determine whether the sexes respond differently to calorie and macronutrient manipulations, particularly in terms of lifespan and reproduction.

An additional complication in the debate over the importance of calories versus macronutrient ratio, and sex-specific effects, is the focus on a small number of laboratory model species (yeast, nematode worms, drosophila, mice and rats, (see Nakagawa et al., 2012, Moatt et al., 2016 and Chapter 2) to study these effects. Recent meta-analytic insights suggest that DR is twice as effective at extending lifespan in these model species as in other species, possibly due a faster rate of reproductive decline in response to DR in model species (Nakagawa et al., 2012, Moatt et al., 2016; Chapter 2). The majority of evidence against the role macronutrients comes from vertebrate studies, leading to the suggestion of species specificity in the action of DR, with caloric restriction being more effective in vertebrates and macronutrient manipulations more effective in invertebrates (Speakman et al., 2016). However, the majority of studies comparing caloric restriction to macronutrient content in vertebrates have used laboratory strains of mice (e.g. Mitchell et al., 2015a, Mitchell et al., 2015b, Mitchell et al., 2015c and see Speakman et al., 2016 for review), whereas a much greater variety of invertebrate species have been tested (e.g , fruit flies (Lee et al., 2008, Lee, 2015, Jensen et al., 2015); crickets (Maklakov et al., 2008, Maklakov et al., 2009); tephritid fruit flies (Carey et al., 2008); and Queensland fruit flies (Fanson et al., 2009, Fanson et al., 2012). Combined with the lack of studies implementing the GF, this focus on model

species in vertebrates makes general conclusions about the role of macronutrients versus calories difficult to draw.

Here we address these issues by applying the GF to distinguish the effects of dietary macronutrient and caloric content on lifespan and reproduction in a wild-derived population of three-spined sticklebacks (*Gasterosteus aculeatus*). Specifically we address the following outstanding issues: 1) is calorie intake or macronutrient ratio the key determinant of mortality risk in a non-model vertebrate species; 2) do calories or macronutrients have a greater impact on reproductive performance and 3) are there sex differences in the response to DR when males experience a greater range of reproductive costs. Importantly, we manipulate calories by restricting dietary availability (i.e. restriction) rather than via dilution (see methods). Given recent evidence, we predict that lifespan will be maximised on low protein : lipid intakes. Correspondingly we expect reproduction will be maximised on high protein : lipid intakes. However, we expect to see sex specific optima with regard to which intakes maximise reproduction and lifespan, and therefore fitness.

4.3 Materials and Methods

4.3.1 Husbandry

Experimental individuals were first generation offspring of wild caught three-spine sticklebacks. Parent fish were collected from Inverleith Pond, Edinburgh (55.96N 3.22W) during the spring of 2014. Using standard IVF techniques (Barber and Arnott, 2000), 23 clutches were produced, each from a unique sire and dam. Offspring were maintained on a diet of live artemia and fry powder (ZM Sytems, ZM-100 Fry Food: protein 55.0%, oil 13.0% and ash 12.0%), until three months of

age. From three to four months of age (the start of dietary manipulations), fish were fed standard grade fish pellet (ZM Systems, medium granular: protein 52.0%, oil 12.0% and ash 10.3%) to condition them to surface feeding. At four months of age fish were sexed molecularly through fin clips and weighed, then assigned to one of 15 diet treatments. Initially, a total of 600 individuals, 300 of each sex, were split equally across diets (n= 20 per sex, per dietary treatment). However, due to mortality prior to the start of dietary manipulations, a total of 594 individuals were used in the experiment (see Appendix 3: Table S3.1 for breakdown of sample sizes).

Fish were housed in flow through glass sided aquaria split into compartments (7 x 25 x 50cm) housing a single fish and containing an artificial plant. Compartments were created using opaque semi-permeable plastic divides, allowing water movement but preventing the movement of fish or food debris. Temperature and light regimes were matched to the natural levels for Edinburgh at that time of year. Clutches and treatments were evenly distributed between stacks and shelves to control for both family and tank effects.

4.3.2 Dietary treatments

The composition of diets used in this experiment were the same as those used in Chapter 3 (see Appendix 3: Table S3.2). Unlike rodents and insects, where studies focus on the ratio of protein : carbohydrate ingested (e.g Lee et al., 2008, Maklakov et al., 2008), carbohydrate is not a key macronutrient for predatory fish (Ruohonen et al., 2003, Ruohonen et al., 2007), thus we are able to vary the ratio of protein : lipid in the diet, without having to control for carbohydrate intake. Therefore, a total of five diets were used which varied in the ratio of protein to lipid (Table 4.1). To

achieve this range of diets and to reduce the correlation between protein and lipid content (see Appendix 3: Fig. S3.1), we used inert carbohydrate filler. This is indigestible in teleosts (Kim and Kaushik, 1992, Guillaume, 2001) and thus, although the diets differ in carbohydrate content (Table 4.1), this is indigestible filler. All diets were designed to have an excess of carotenoid so that individuals on the smallest ration would ingest sufficient carotenoid to display nuptial colour (see below).

Table 4.1 Table of the nutrient content of the five diets used in this experiment. Note, carbohydrate in these diets is indigestible filler.

Protein (%)	Lipid (%)	Carbohydrate (%)	Ratio P:L	Calories (MJ/kg)
67.5	6.6	15.8	10.2 : 1	19.3
33.2	3.9	53.1	8.5 : 1	17.5
59.3	13.0	16.1	4.6 : 1	20.2
51.6	20.5	17.8	2.5 : 1	22.2
31.2	19.2	39.7	1.6 : 1	21.5

Each of the five diets were provided at one of three levels; 100% (*ad libitum*), 75% and 50% of *ad lib*, giving a total of 15 dietary treatments. In the majority of studies that vary macronutrient ratio, food is provided *ad lib*, requiring food to be available at all times. However, to avoid problems associated with dietary dilution and the additional problem of rapid food degradation in water, we used an intermittent feeding regime which has previously been used successfully to implement DR in fish (e.g. Terzibasi et al., 2009 and Chapter 3). Individuals in the 100% treatment were fed twice a day, the 75% treatment were fed alternately once a day and then twice on the second day and the 50% treatment were fed once a day.

Feeding levels were quantified using monthly monitoring of sentinel fish for each diet from the 100% treatment (see Appendix 3). To account for differences in feeding rate between fish of different size, we classified fish of each treatment as either large (heaviest 10 fish) or small (lightest 10 fish) for each sex. Thus there were 60 different feeding quantities (sex*diet*level*size combination) each being fed to 10 individuals (see Appendix 3: Table S3.1). Diets were initiated 24/11/2014 when fish were 172.24 ± 0.08 days old and individuals were maintained on diets for life.

Data Collection

4.3.3 Mortality

Fish were checked twice daily for their survival status and date of death was recorded. For welfare reasons, any individual showing signs of ill health for two consecutive days were humanely sacrificed (see Appendix 3 for more details). On day 749 of the experiment only 53 individuals remained alive (46 males and 7 females). For logistical reasons, these individuals were culled using standard approved procedures in the UK.

4.3.4 Reproductive Investment

In the wild, sticklebacks typically undergo a single breeding season per year, commencing during the early spring and lasting through to late summer or early autumn (Wootton, 1984). However, under laboratory conditions, sticklebacks can undergo a second breeding season, though typically this involves a small number of individuals and limited reproduction (e.g. Inness and Metcalfe, 2008). A number of fish survived to a second breeding season in our experiment and although we recorded any reproductive activity, not enough individuals attempted to reproduce to

allow statistical analysis (see Appendix 3). Therefore, all reproductive data presented here is from the first season, which represents a good proxy for lifetime reproductive investment.

Nuptial Colour

During the breeding season, male three-spine sticklebacks develop red nuptial colouration (Wootton, 1984). From the start of the male breeding season (defined as > 20% of males having developed nuptial colouration), monthly photographs of males were taken using standard procedures (see Frischknecht, Braithwaite and Barber, 2000, Barber et al., 2001 and Appendix 3). Photographs continued until the breeding season ended, defined as when < 20% of males expressed colour. Photographs were analysed using ImageJ software (see Appendix 3 for full methods). From these we recorded intensity and area covered of both red and blue colour. Red intensity was standardised by dividing by values obtained from a white colour standard included in all photographs ($red = red_{fish} / white_{standard}$).

Male Reproductive Behaviour

In the wild, male three-spine sticklebacks construct nests from filamentous algae and sediment during the breeding season, in which females lay eggs (Wootton, 1984). Once a male developed nuptial colour, they were provided with nesting material and were stimulated to construct a nest by presenting them with an image of a gravid female once per day, following standard procedure (Barber et al., 2001). To assess nest building we recorded time until nest construction began (days) and the time taken to complete the nest (days, see Appendix 3 for full details). Males were allowed to construct multiple nests throughout the breeding season (see Appendix 3).

If a male failed to start constructing a nest on three consecutive attempts, all nesting material was removed and no further material provided.

When nesting, males defend their breeding territory and actively court females (see Wootton, 1984 and Appendix 3). Within 7 days of a male starting nest construction, male defensive behaviour was recorded by suspending a red object in their tank for a five minute observation period (see Appendix 3 for full procedure). This protocol was repeated for each nest a male initiated. Investment in territory defence was measured as the total time (s) spent defending the nest across all nests. Within 7 days of a nest being completed, male courtship was recorded by presenting each male with an image of a gravid female for a 5 minute observation period (see Appendix 3). Again this was repeated for all nests that were completed and courtship investment was measured as the total time (s) spent courting across all nests.

Female Reproduction

Females are capable of producing multiple egg clutches per breeding season (Wootton, 1984) and females can be stripped of their eggs without causing any ill effects (Barber and Arnott, 2000). We therefore monitored females daily during the course of the breeding season and, when deemed gravid (indicated by a swollen and distended abdomen) removed them from their tank and manually stripped them of eggs using standard techniques (see Barber and Arnott, 2000 and Appendix 3). In brief: the female was dried and weighed, then the expulsion of the egg sack was encouraged by lightly rubbing a finger down both sides of the fish, the female was then reweighed and returned to her tank. The eggs were spread into a monolayer within a petri dish and the eggs counted twice, the egg number was recorded as the

average between these two numbers (to the nearest whole number). This was repeated whenever females' were deemed to be gravid. We therefore quantified: the total number of eggs produced by each female, the total number of egg clutches produced by a female and the average number of eggs per clutch. Thus we can distinguish whether any differences in lifetime egg production are due to females producing more clutches, more eggs per clutch or both. Occasionally, a female would lay eggs naturally before they could be manually stripped (N=210 out of 2766 clutches). To allow us to get a measure of total number of eggs produced, a natural laying event was given an average egg score (mean number of eggs per clutch for that female).

4.3.5 Statistical Analysis

All analyses were carried out in R (v3.4.0, R core team, 2017). We used a multivariate response-surface approach (Lande and Arnold, 1983) to estimate the linear and non-linear effects (quadratic and correlation) of protein and lipid intake and the interaction between them on our response variables (Lee et al., 2008, Maklakov et al., 2008, Maklakov et al., 2009, Fanson et al., 2009, Solon-Biet et al., 2014, Jensen et al., 2015 and see below). As recommended (Lande and Arnold, 1983) estimates of linear terms were taken from a model only containing linear terms whereas estimates of non-linear terms were taken from a model including linear and non-linear terms. For all analyses, protein and lipid intakes were standardised to a mean of zero and a standard deviation of one to avoid issues of scale differences when fitting quadratic terms. For all traits we performed separate analyses for each sex to test for sex-specific effects of macronutrients. We then combined the data and performed a full analysis with sex interacted with protein and lipid to test if the effect

of macronutrients differed between the sexes. For models comparing reproductive investment between the sexes, response variables were also standardised to a mean of zero and a standard deviation of one to facilitate comparison. Nutritional landscapes were visualised using thin-plate splines from the package *fields* (Nychka et al., 2015).

Survival was analysed via generalised linear mixed models (GLME) in the package *Lme4* (Bates et al., 2015), implementing an event history analysis (e.g. Therneau and Grambsch, 1991, Thomson et al., 2017). Previous similar experiments have analysed lifespan against intake rates measured from a period when growth has ceased and thus intake rates are stable (e.g. Solon-Biet et al., 2014). However, sticklebacks have indeterminate growth and thus intake rates change over time, making this approach unfeasible. Event history models allow for time varying covariates. We therefore analyse mortality as a weekly event. Individuals' were assigned a weekly survival value (0 = survived, 1 = death), which represents the response variable. This was then modelled against the linear and quadratic effects of protein and lipid intake for that week. These models also included initial weight as a continuous covariate to control for differences in mortality due to size that were not determined by diet. Visual inspection of mortality risk revealed clear variation across the study period (see Appendix 3: Fig. S3.2). We therefore subdivided the experiment into six periods where the mortality risk was noticeably different to assess whether the impact of intake on mortality risk varied across these periods (see Appendix 3: Fig. S2). Models also included experimental week and individual identity as random effects to allow for heterogeneity in mortality risk across time and

individuals. The 53 individuals that survived to the end of the experiment were included as censored data points, never having a weekly survival of 1.

Measures of total reproductive investment (total time courting, total time defending breeding territory and total number of eggs produced) were analysed using linear mixed effects models (LME). The linear and quadratic effects of protein and lipid were included as continuous covariates, with shared tank and family group included as random effects. As intake rates were stable throughout the breeding season, we analysed the average daily intake ($gday^{-1}$) of protein and lipid across the course of the breeding season.

As we monitored both male and female reproduction at multiple time points throughout the breeding season (see above), we also investigated reproductive senescence in both sexes. We used LME models to explore the effects of protein and lipid intake on age-specific reproduction in both sexes (Maklakov et al., 2009, Jensen et al. 2015). We analysed the number of eggs produced for females and the time courting for males at each time point. We include courtship rather than territory defence as the overall effects of macronutrient intake was the same for both traits (see below). We fitted both the linear and non-linear effects of age, age of first reproductive event, age of last reproductive event, protein intake and lipid intake, with individual ID included as a random effect. This approach accounts for within and between individual variation in age-specific reproduction (Van De Pol and Verhulst, 2006). Previous work used lifespan rather than age of last reproductive event (see Maklakov et al., 2009, Jensen et al., 2015), however here, a large number of individuals survived long after they stopped reproducing. Therefore, we use age of last reproductive event, as we felt this was a more appropriate measure than lifespan.

Estimates for the linear effects of protein and lipid were taken from a model including both the linear and non-linear effect of age, as the effect of age was strongly non-linear. In addition to courtship we also investigated age specific investment in nuptial colouration for males. This was not included in measures of total reproduction as there is no obvious way of summing measures of colouration to create a cumulative measure. All males were photographed at the same time points, we therefore do not include age of first reproductive event. However, because males die during the breeding season, we did include age of last record of colouration to account for selective disappearance from the population.

4.4 Results

4.4.1 Mortality

Our results suggest that mortality risk was significantly affected by nutrient intake and this varied between the sexes. In both sexes, time period significantly affected mortality risk (Males: $\chi^2 = 89.15$; $p < 0.001$; Females: $\chi^2 = 78.38$; $p < 0.001$; Appendix 3: Tables S3.3 and S3.4, Fig. S2), with risk fluctuating over time in males, but generally increasing over time in females. In males increasing lipid intake reduced mortality risk in a non-linear manner, which remained consistent throughout the course of the experiment (GLME; Lipid: $\chi^2 = 9.47$; $p = 0.002$; Lipid^2: $\chi^2 = 4.32$; $p = 0.038$; Time period*Lipid: $\chi^2 = 7.78$; $p = 0.168$; Time period*Lipid^2: $\chi^2 = 1.79$; $p = 0.877$; Fig. 4.1; Table 4.2; Appendix 3: Table S3.3). However, there were no-significant effects of protein on male mortality risk (GLME; Protein: $\chi^2 = 2.87$; $p = 0.090$; Protein^2: $\chi^2 = 2.20$; $p = 0.138$; Fig. 4.1; Appendix 3: Table S3.3). As can be seen from Fig. 4.1A there is no effect of caloric intake on male mortality risk, as

decreasing caloric intake often increases mortality risk. There was a marginally non-significant effect of initial weight on male mortality risk (GLME; $\chi^2 = 3.29$; $p = 0.070$; Appendix 3: Table S3.3). The effect of macronutrients differed for females. Although there was no overall effect of protein on mortality risk, there was a significant interaction between time period and protein on mortality risk (GLME; Time period*Protein: $\chi^2 = 16.16$; $p = 0.006$; Protein: $\chi^2 = 0.12$; $p = 0.733$; Protein^2: $\chi^2 = 0.31$; $p = 0.576$; Fig. 4.1; Table 4.3; Appendix 3: Tables S3.4). This suggests that the effect of protein varies, for example significantly reducing mortality risk in period 1 (Fig. 1B) yet becoming non-significant and slightly increasing mortality risk in period 5 (Fig.1C). However, there was no effect of lipid intake on female mortality risk (GLME; Lipid: $\chi^2 < 0.00$; $p = 0.981$; Lipid^2: $\chi^2 = 0.31$; $p = 0.575$; Fig. 4.1; Appendix 3: Table S3.4). As with males, there was no effect of caloric intake on female mortality risk (Fig. 4.1 B & C). Although in time period 5 (Fig. 4.1C) it does appear that reducing intake improves mortality risk, this is clearly driven by protein intake with the effect of caloric intake being much weaker. There was and no effect of initial weight on female mortality risk (GLME; $\chi^2 = 0.02$; $p = 0.894$; Appendix 3: Table S3.4).

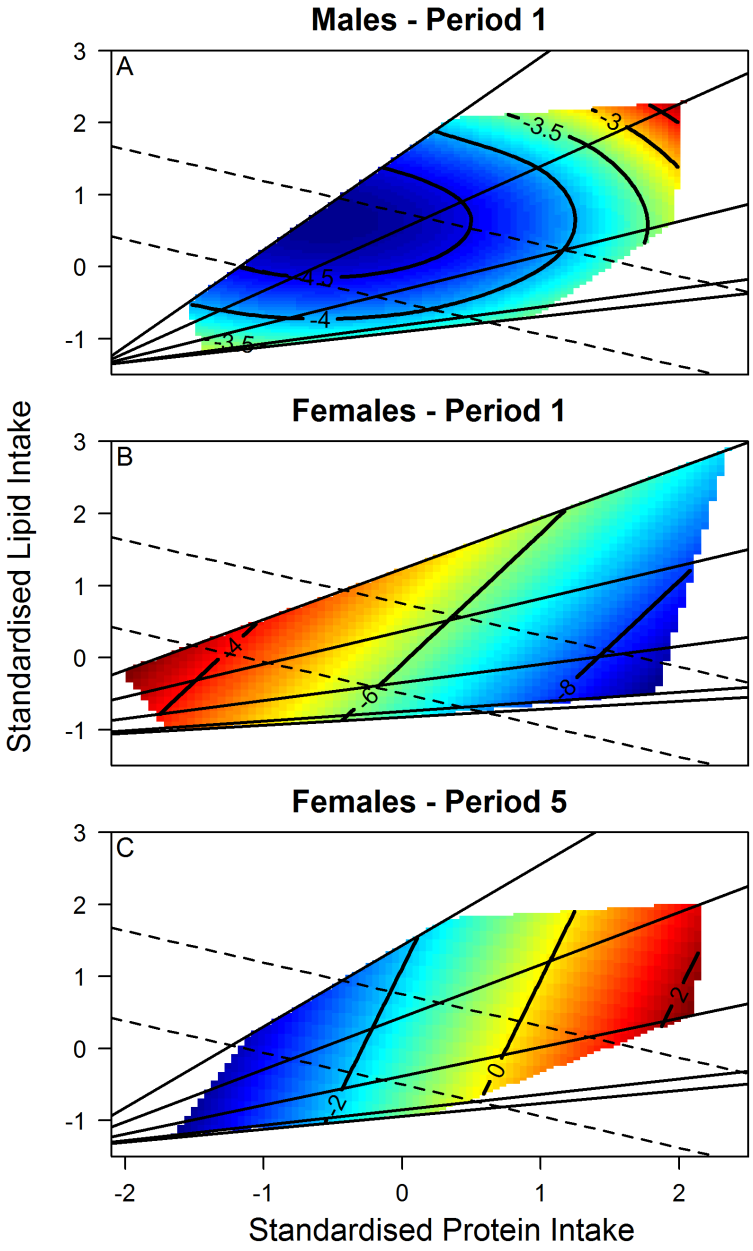

Figure 4.1 Non-parametric thin-plate spline contour visualisations showing the effects of protein and lipid intake on mortality risk, (A) represents the effect in males in time period 1, (B) represents the effect in time period 1 for females and (C) represents the effect in time period 5 for females. Positive values suggest high risk, negative values suggest low risk. Mortality risks are calculated from model outputs and hypothetical intakes (standardised to a mean of zero and s.d. of 1). The five solid lines originating from the y-axis represent the 5 ratios of protein : lipid used in this experiment, the dashed lines represent isocaloric intakes. There was a significant non-linear effect of lipid on male mortality risk ($p = 0.008$) for all time periods. In females there was an effect of protein on mortality risk however this changed over time, for example being beneficial in time period 1 (B) and detrimental in period 5 (C).

Table 4.2 Outputs from event history model (binomial GLME) exploring Male mortality risk. Model contains main effects that were significant in previous models (Appendix 3: Table S3.3) and their interactions.

	Estimate (± s.e.)	χ^2	***p***
intercept	-4.684 (0.311)		
Time Period 2	-0.847 (0.344)		
Time Period 3	0.812 (0.349)		
Time Period 4	-0.162 (0.385)		
Time Period 5	2.444 (0.741)		
Time Period 6	1.159 (1.167)	88.16	< 0.001
Lipid	-1.590 (0.631)	10.25	0.001
Lipid^2	1.212 (0.794)	7.07	0.008
Time Period 2*Lipid	-0.283 (0.965)		
Time Period 3*Lipid	-0.146 (0.863)		
Time Period 4*Lipid	0.236 (0.875)		
Time Period 5*Lipid	0.325 (1.172)		
Time Period 6*Lipid	2.987 (1.927)	7.78	0.168
Time Period 2*Lipid^2	0.397 (1.060)		
Time Period 3*Lipid^2	0.241 (0.959)		
Time Period 4*Lipid^2	-0.006 (0.989)		
Time Period 5*Lipid^2	0.892 (2.111)		
Time Period 6*Lipid^2	-3.741 (3.570)	1.79	0.877

Table 4.3 Outputs from event history model (binomial GLME) exploring Female mortality risk. Model contains main effects that were significant in previous models (Appendix 3: Table S3.4) and their interactions.

	Estimate (± s.e.)	χ^2	*p*
intercept	-5.927 (0.617)		
Time Period 2	0.814 (0.620)		
Time Period 3	2.594 (0.643)		
Time Period 4	2.074 (0.693)		
Time Period 5	4.480 (0.880)		
Time Period 6	1.311 (1.734)	78.36	< 0.001
Protein	-1.466 (0.722)	0.14	0.707
Lipid	0.810 (0.444)	0.03	0.866
Time Period 2*Lipid	-1.036 (0.479)		
Time Period 3*Lipid	-0.739 (0.456)		
Time Period 4*Lipid	-0.978 (0.470)		
Time Period 5*Lipid	-0.498 (0.592)		
Time Period 6*Lipid	-0.492 (0.997)	7.15	0.210
Time Period 2*Protein	1.966 (0.751)		
Time Period 3*Protein	1.454 (0.730)		
Time Period 4*Protein	1.420 (0.732)		
Time Period 5*Protein	1.921 (0.856)		
Time Period 6*Protein	-1.736 (1.795)	16.16	0.006

Comparing the Sexes

Cross sex comparisons suggest that mortality risk differed between the sexes across time periods (GLME; Period*Sex: $\chi^2 = 42.40$; $p < 0.001$; Table 4.4). Similarly, the beneficial effect of lipid is stronger in males than in females, with a significant sex by lipid interaction (GLME; $\chi^2 = 4.95$; $p = 0.026$; Table 4.4). Despite the suggestion of an effect of protein on female but not male mortality, there was no

evidence of a difference in the effect of protein between the sexes (GLME; $\chi^2 = 1.11$; $p = 0.292$; Table 4.4). However, power issues prevented us from fitting the three way interaction between time period, sex and protein intake. As the effect of protein in females was only present in some time periods, it is possible that there are sex differences in the effect of protein, but only in certain time periods, and that a bigger dataset would allow us to pick these up.

Table 4.4 Outputs from event history model (binomial GLME) exploring differences in mortality risk between the sexes. Model contains main effects that were significant in split sex models (see above) and their interactions.

	Estimate (± s.e.)	χ^2	***p***
intercept	-5.165 (0.293)		
Time Period 2	0.087 (0.315)		
Time Period 3	1.879 (0.343)		
Time Period 4	1.442 (0.380)		
Time Period 5	3.423 (0.466)		
Time Period 6	3.815 (0.629)		
Protein	0.021 (0.100)		
Lipid	-0.073 (0.241)		
Lipid^2	0.067 (0.218)		
Sex (male)	0.766 (0.295)		
Protein*Sex	0.138 (0.156)	0.79	0.373
Lipid*Sex	-1.02 (0.379)	3.83	0.050
Lipid^2*Sex	0.754 (0.353)	4.66	0.031
Time Period 2*Sex	-1.198 (0.383)		
Time Period 3*Sex	-1.419 (0.365)		
Time Period 4*Sex	-1.945 (0.368)		
Time Period 5*Sex	-1.867 (0.37)		
Time Period 6*Sex	-2.573 (0.516)	43.59	< 0.001

4.4.2 Reproductive Investment

Male Reproductive Behaviour

Male reproductive behaviour was strongly influenced by protein intake. There was a positive linear effect of protein, with higher protein intakes resulting in greater courtship investment (LME; Protein: $\chi^2 = 6.72$; $p = 0.010$; Fig. 2; Table 4.5). There was a marginally non-significant quadratic effect of lipid (LME: Lipid^2: $\chi^2 = 3.52$; $p = 0.061$; Fig. 4.2; Table 4.5), but no significant linear effect (LME; Lipid: $\chi^2 = 0.71$; $p = 0.397$; Fig. 4.2; Table 4.5). All other non-linear effects were non-significant (all $p > 0.2$; Table 4.5). The same general patterns were observed for territory defence, with total time spent defending the nest increasing with increasing protein intake (LME; $\chi^2 = 6.66$; $p = 0.010$; Fig. 4.2; Table 4.5) and all other effects being non-significant (all $p > 0.1$; Table 4.5). The general patterns observed here hold true for a number of other potential measures of both of these traits (see Appendix 3: Tables S3.5 and S3.6). In contrast, there was no suggestion of an effect of macronutrient intake on the number of nests attempted (all $p > 0.08$; Appendix 3: Table S3.7) or the number of nest completed (all $p > 0.1$; Appendix 3: Table S3.7).

Female Reproductive Investment

Female reproduction was strongly influenced by the intake of both protein and lipid. There was a positive linear effect of protein, with increasing protein intake increasing total egg production (LME; $\chi^2 = 10.93$; $p < 0.001$; Fig. 4.2; Table 4.5). However, the effect of lipid intake was non-linear, with egg production highest at intermediate intakes (LME; Lipid^2: $\chi^2 = 12.27$; $p < 0.001$; Lipid: $\chi^2 = 1.64$; $p = 0.200$; Fig. 4.2; Table 4.5). All other non-linear effects were non-significant (all $p > 0.1$;

Table 4.5). This increase in total egg production was due to an increase in both the size and number of clutches produced by females on high protein and intermediate lipid intakes (see Appendix 3: Table S3.8). The same general pattern was observed for all female reproductive traits we analysed (see Appendix 3: Table S3.9).

Table 4.5. Outputs from LME models exploring the linear and non-linear effects of protein and lipid on reproductive investment. Courtship effort is total time spent courting (s), territory defence is total time spent defending the nest (s) and total egg production is the total number of eggs produced across breeding season one.

	Estimate (± s.e.)	χ^2	***p***
Courtship Effort			
Protein	86.05 (32.90)	6.72	0.010
Lipid	-28.50 (33.98)	0.71	0.397
Protein^2	-233.94 (212.61)	1.22	0.269
Lipid^2	-347.51 (183.45)	3.52	0.061
Protein*Lipid	61.25 (70.14)	0.76	0.383
Territory Defence			
Protein	76.95 (29.45)	6.66	0.010
Lipid	-31.97 (30.31)	1.12	0.291
Protein^2	-156.34 (192.74)	0.67	0.412
Lipid^2	-217.04 (164.76)	1.72	0.189
Protein*Lipid	56.15 (63.15)	0.80	0.371
Total Egg Production			
Protein	156.38 (46.12)	10.93	< 0.001
Lipid	58.29 (46.02)	1.64	0.200
Protein^2	11.95 (274.19)	< 0.00	0.960
Lipid^2	-906.17(257.99)	12.27	< 0.001
Protein*Lipid	119.07 (87.91)	1.86	0.172

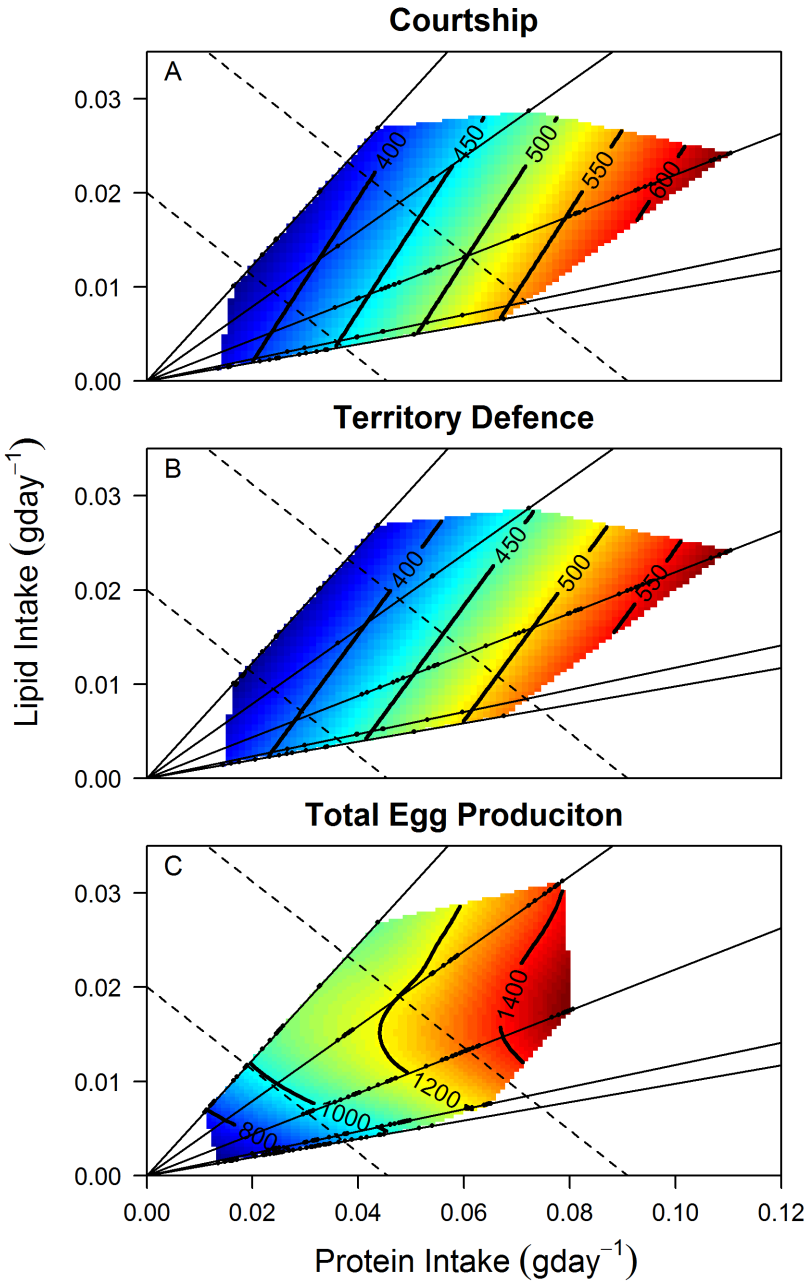

Figure 4.2 Non-parametric thin-plate spline contour visualisations showing the effects of protein and lipid intake on: (A) Courtship (time courting (s)), (B) Territory Defence (time displaying (s)) and (C) Total Egg Production. The five solid lines originating from the origin represent the 5 ratios of protein : lipid used in this experiment, the dashed lines represent isocaloric intakes. In general, there was a positive linear effect of protein and a non-linear effect of lipid on the total number of eggs produced by a female. In males there was a positive linear effect of protein on reproductive behaviour (territory defence and courtship).

Comparing the Sexes

For comparison between the sexes, we used courtship as our measure of male reproduction, as the effect of macronutrients were similar for both courtship and territory defence. Increasing protein intake had the same beneficial effect on reproduction for both sexes (LME; Sex*Protein: $\chi^2 = 1.04$; $p = 0.307$; Sex*Protein^2: $\chi^2 = 0.82$; $p = 0.366$; Table 4.6). Despite lipid having no effect in males but a non-linear effect in females, there was no evidence that the effect of lipid differed between the sexes (LME; Sex*Lipid: $\chi^2 = 1.39$; $p = 0.239$; Sex*Lipid^2: $\chi^2 = 0.00$; $p = 0.973$; Table 4.6).

Table 4.6 Outputs from minimal LME model exploring the sex differences in the effects of macronutrient on reproduction. Female reproduction = total egg production, male reproduction = total courtship (s).

	Estimate (± s.e.)	χ^2	***p***
intercept	0.014 (0.082)		
Protein	-0.093 (0.304)		
Lipid	0.543 (0.235)		
Protein^2	0.189 (0.309)		
Lipid^2	-0.492 (0.235)		
Sex (male)	-0.033 (0.088)		
Protein*Sex	0.462 (0.410)	1.04	0.307
Protein^2*Sex	-0.370 (0.412)	0.82	0.366
Lipid*Sex	-0.134 (0.356)	1.39	0.239
Lipid^2*Sex	0.010 (0.357)	0.00	0.973

4.4.3 Reproductive Senescence

Courtship investment

There was a significant non-linear effect of age on courtship in males, with investment in courtship increasing initially but declining at older ages (LME; Age^2: $\chi^2 = 22.95$; $p < 0.001$; Age: $p < 0.001$; Fig 4.3; Appendix 3: Table S3.9). Despite a positive linear effect of protein on investment on courtship (see above), there was no effect of protein on reproductive senescence (LME; Age*Protein: $\chi^2 = 0.00$; $p = 0.946$; Fig 4.3; Table 4.7). There was also no effect of lipid on reproductive senescence (LME; Age*Lipid: $\chi^2 = 0.00$; $p = 0.945$; Fig 4.3; Table 4.7). However, there was a negative linear effect of age of first reproductive event on investment, with those males starting reproducing later in life having lower investment in courtship (LME; Age First: $\chi^2 = 9.39$; $p = 0.002$; Appendix 3: Table S3.9).

Table 4.7 Outputs from LME models of reproductive senescence in male courtship (time spent courting (s)). Model contains main effects that were significant in previous models (Appendix 3: Table S3.9) and their interactions.

	Estimate (± s.e.)	**χ^2**	***p***
intercept	-0.048 (0.074)		
Age	1.168 (0.229)		
Age^2	-1.135 (0.229)		
Age First	-0.741 (0.307)		
Protein	0.126 (0.049)		
Lipid	-0.085 (0.050)		
$Age\ First^2$	0.598 (0.296)		
Age*Protein	-0.002 (0.031)	0.00	0.946
Age*Lipid	0.002 (0.032)	0.00	0.945

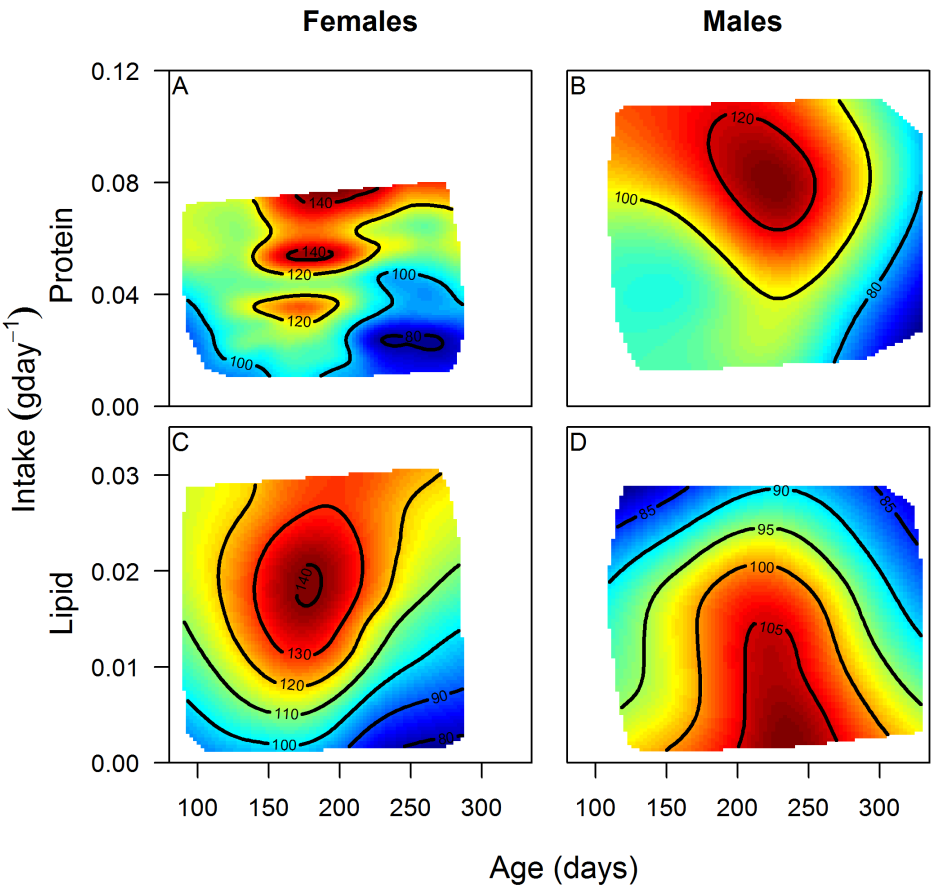

Figure 4.3 Non-parametric thin-plate spline contour visualisations showing the effects of protein and lipid intake on reproductive senescence. Panel response surfaces are as follows: (A) and (C) female egg production (number of eggs produced at each clutch), (B) and (D) male courtship (time spent courting (s)). All plots have age on the x axis with (A and B) having protein (gday^{-1}) and (C and D) having lipid (gday^{-1}) on the y axis.

Nuptial Colour

There was also a significant non-linear effect of age on male nuptial colour, with red intensity increasing to a peak at the height of the breeding season then declining towards the end (LME; Age^2: $\chi^2 = 593.06$; $p < 0.001$; Age: $p < 0.001$; Appendix 3: Table S3.10, Fig. S3.3). There was a significant non-linear effect of lipid intake, with red intensity highest at intermediate intakes (LME; $Lipid^2$: $\chi^2 = 8.40$; $p = 0.004$; Lipid: $\chi^2 = 3.95$; $p = 0.047$; Appendix 3: Table S3.10, Fig. S3.3). However, there was no evidence of an interaction between lipid and age, suggesting that lipid intake did not alter the effect of age on nuptial colouration (LME; $\chi^2 = 0.00$; $p = 0.989$; Table 4.8; Appendix 3: Fig. S3.3). There was no overall effect of protein intake on red intensity (LME; Protein: $\chi^2 = 0.61$; $p = 0.435$; $Protein^2$: $\chi^2 = 2.57$; $p = 0.109$; Appendix 3: Table S3.10, Fig. S3.3) and no effect of protein on the change in nuptial colouration with age (Age*Protein: $\chi^2 = 2.69$; $p = 0.101$; Table 4.8; Appendix 3: Fig. S3.3). There was a positive interaction between protein and lipid intake, suggesting the beneficial effect of lipid was stronger with higher protein intakes (Protein*Lipid: $\chi^2 = 5.49$; $p = 0.019$; Appendix 3: Table S3.10). Finally there was a marginally non-significant positive linear effect of age of last measurement, suggesting longer living males had slightly higher red intensity (Last: $\chi^2 = 3.24$; $p = 0.071$; Appendix 3: Table S3.10).

Table 4.8. Outputs from LME model exploring senescence of male nuptial colour (red intensity). Model contains main effects that were significant in previous models (Appendix 3: Table S3.10) and their interactions.

	Estimate (± s.e.)	χ^2	*p*
intercept	0.474 (0.005)		
Age	0.257 (0.009)		
Age^2	-0.259 (0.009)		
Age Last	0.003 (0.002)		
Protein	< 0.000 (0.003)		
Lipid	0.029 (0.010)		
$Lipid^2$	-0.025 (0.010)		
Protein*Lipid	0.004 (0.003)		
Age*Lipid	< -0.00 (0.002)	0.00	0.989
Age*Protein	0.002 (0.001)	2.69	0.101

Female egg production

Similar to male nuptial colour and courtship investment, there was a non-linear effect of age on female reproductive investment, with clutch size increasing to a peak at intermediate ages and then declining in old age (LME; Age^2: $\chi^2 = 161.86$; $p < 0.001$; Age: $\chi^2 = 108.99$; $p < 0.001$; Fig 4.3; Appendix 3: Table S3.11). There was a significant effect of protein intake on this reproductive decline, with high protein intakes resulting in a slower rate of decline in clutch size (LME; Age*Protein: $\chi^2 = 13.49$; $p < 0.001$; Fig 4.3; Table 4.9), however there was no effect of lipid on senescence (LME; Age*Lipid: $\chi^2 = 2.22$; $p = 0.136$; Fig 4.3; Table 4.9). There was a negative linear effect of age of first reproductive attempt on subsequent reproduction, meaning those individuals starting to reproduce later in life produced smaller clutches (LME; $\chi^2 = 9.00$; $p = 0.002$; Appendix 3: Table S3.11). There was a significant effect of age of last reproductive attempt on clutch size, suggesting that individuals reproducing later in life overall produced larger clutches (LME; Age

Last: $\chi^2 = 1.14$; $p = 0.285$ Appendix 3: Table S3.11). There was a positive interaction between age of first reproduction with age (LME; Age*Age First: $\chi^2 = 13.14$; $p < 0.001$; Fig. 4.4A; Table 4.9), suggesting that those individuals starting reproduction early in life produced larger clutches but suffered a faster rate of reproductive senescence in later life (see Fig. 4.4A). Similarly there was an interaction between age of last reproduction with age (LME; Age*Age Last: $\chi^2 = 13.11$; $p < 0.001$; Fig. 4.4B; Table 4.9), this shows that individuals reproducing later in life have a faster rate of increasing clutch size in early life, however, they also suffer a greater decline in reproduction late in their reproductive life (see Fig. 4.4B).

Table 4.9. Outputs from LME models of reproductive senescence in female egg production. Model contains main effects that were significant in previous models (Appendix 3: Table S3.11) and their interactions.

	Estimate (± s.e.)	**χ^2**	***p***
intercept	-0.208 (0.084)		
Age	1.857 (0.146)		
Age^2	-2.038 (0.147)		
Age First	-0.126 (0.040)		
Age Last	0.078 (0.037)		
Protein	0.703 (0.278)		
Lipid	1.407 (0.251)		
$Protein^2$	-0.646 (0.299)		
$Lipid^2$	-1.249 (0.280)		
Protein*Lipid	0.217 (0.097)		
Age*Age First	0.061 (0.017)	13.14	< 0.001
Age*Age Last	0.072 (0.020)	13.11	< 0.001
Age*Protein	0.074 (0.020)	13.89	< 0.001
Age*Lipid	0.028 (0.019)	2.22	0.136

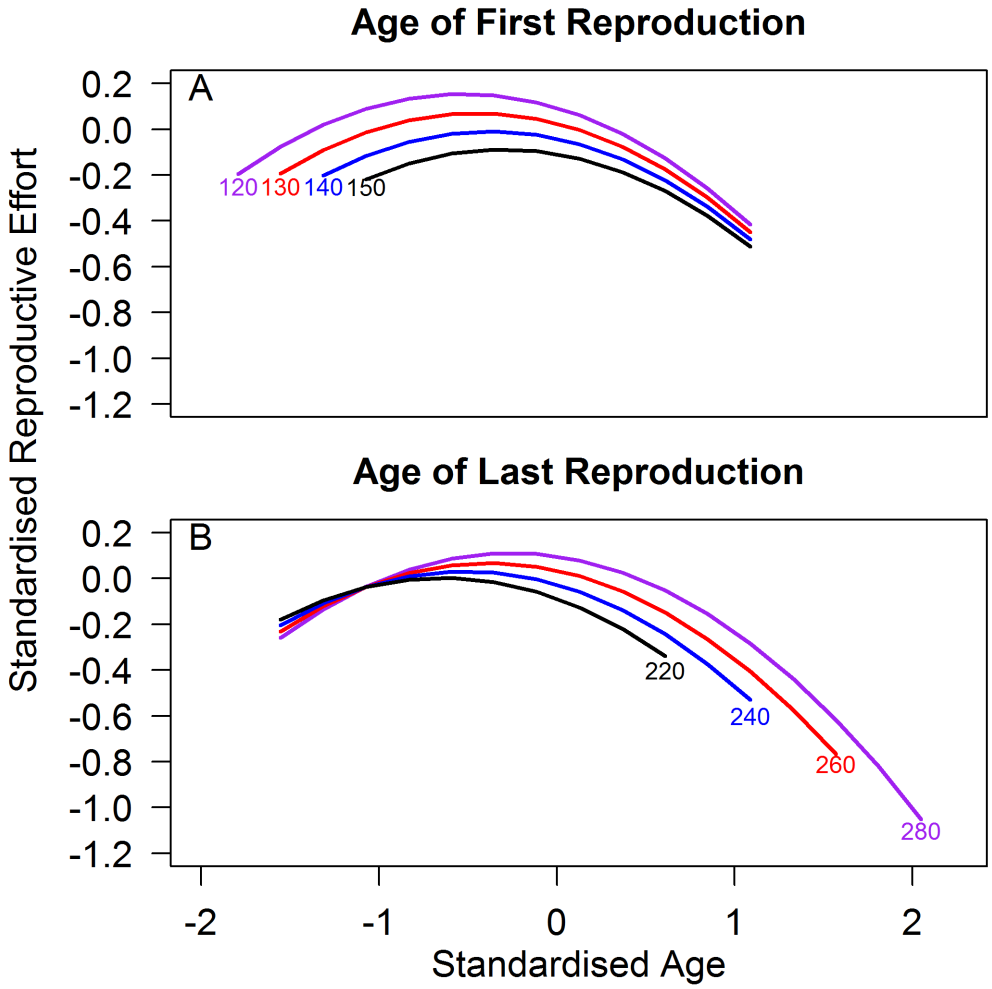

Figure 4.4 Predicted age-specific trajectories of female reproductive senescence. Curves represent the predicted reproductive effort for groups of individuals with different ages of first (A) and last (B) reproductive events (ages indicated by numbers next to the curves). There was a significant effect of both age of first reproduction ($p < 0.001$) and age of last reproductive event ($p < 0.001$) on patterns of female reproductive senescence. The ages selected for each plot were chosen to cover the 90th percentile of the data. For panel (A) the senescence lines end at the mean age of last reproductive event, for panel (B) the curves start at the mean age of first reproductive event. Age and reproductive effort are standardised values (mean of 0 and standard deviation of 1)

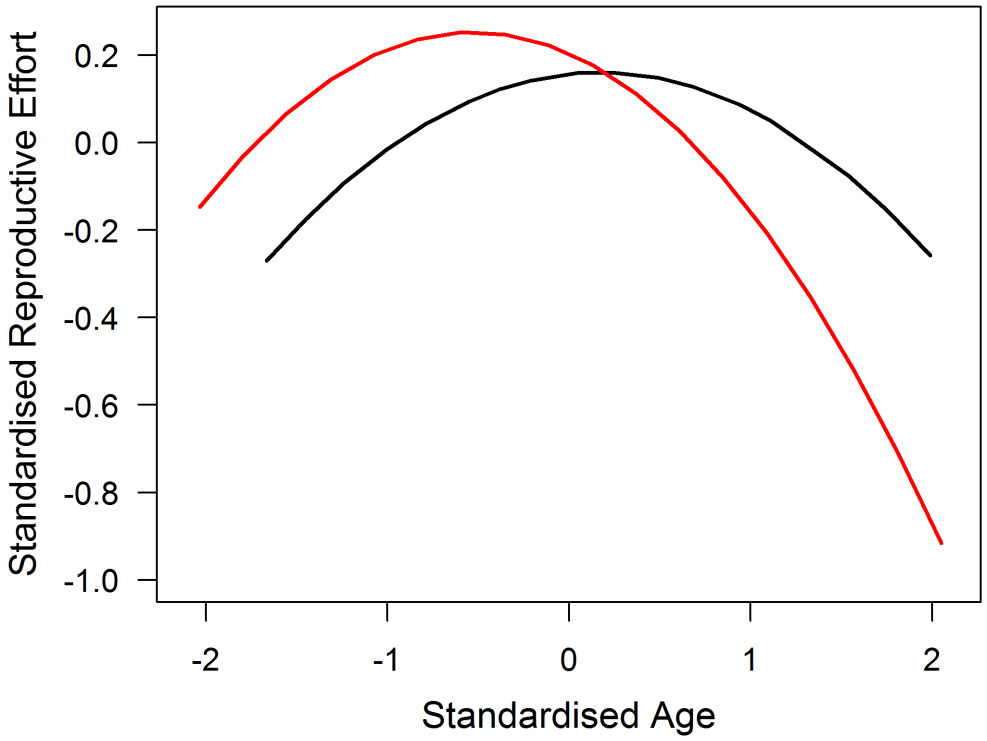

Figure 4.5 Sex-specific patterns of reproductive senescence. There were significant differences is the pattern of reproductive senescense between the sexes (age*sex: $p <$ 0.001, age^2*sex p = 0.051) Females (red) have a higher initial reproductive effort than males (black), however they suffer a much faster rate of reproducitve senescence than males. The age ranges used for these curves were chosen to cover the 90th percentile of the data. Age and reproductive effort are standardised values (mean of 0 and standard deviation of 1).

Comparing the Sexes

As there were no effects of macronutrient intake on ageing in nuptial colour expression, we only compare the differences in ageing of courtship investment and egg production. There were significant differences in the patterns of senescence between the sexes (LME; Age*Sex: $\chi^2 = 45.96$; $p < 0.001$; Age^2*Sex: $\chi^2 = 3.80$;

$p = 0.051$; Figure 4.5; Table 4.10), with females having higher initial reproductive effort than males, but suffering a much faster rate of reproductive senescence (see Fig. 4.5). However, there was no difference in the effect of protein or lipid on reproductive senescence between the sexes (LME; Age*Protein*Sex: $\chi^2 = 2.45$; $p = 0.118$; Age*Lipid*Sex: $\chi^2 = 1.42$; $p = 0.234$ Table 4.10). This suggests that although the rate of senescence differs between the sexes, protein and lipid affect this in the same way for both sexes.

Table 4.10. Outputs from LME model exploring sex differences in reproductive senescence. Male values represent total time spent courting (s) and females measures are total egg production. Models contain main effects that split sex models indicated where significant and interactions.

	Estimate (± s.e.)	χ^2	***p***
intercept	0.028 (0.064)		
Age	1.461 (0.129)		
Age^2	-1.639 (0.129)		
Age First	-0.506 (0.218)		
Age Last	-0.594 (0.247)		
Protein	0.119 (0.050)		
Lipid	0.733 (0.119)		
Sex (male)	0.002 (0.059)		
Age $First^2$	0.372 (0.209)		
Age $Last^2$	0.662 (0.256)		
$Lipid^2$	-0.490 (0.112)		
Age*Sex	-0.286 (0.232)	45.96	< 0.001
Age^2*Sex	0.489 (0.233)	3.80	0.051
Age*Protein	0.052 (0.020)	4.44	0.35
Age*Lipid	0.039 (0.020)	2.55	0.110
Protein*Sex	0.025 (0.072)	0.04	0.838
Lipid*Sex	-0.357 (0.070)	26.24	< 0.001
Age*Protein*Sex	-0.052 (0.033)	2.45	0.118
Age*Lipid*Sex	-0.040 (0.034)	1.42	0.234

4.5 Discussion

It is widely accepted that DR increases lifespan and that this effect is stronger in females than males (reviewed Speakman and Mitchell, 2011, Nakagawa et al., 2012, Selman, 2014). However, growing evidence suggests that macronutrient ratio rather than restriction of caloric intake underpins this effect (reviewed Simpson et al., 2017) and that under this scenario sex differences are less pronounced (Maklakov et al., 2008, Jensen et al., 2015). The majority of this evidence comes from studies of insects and the importance of dietary macronutrient ratio has rarely been tested in vertebrates (Solon-Biet et al., 2014). In addition, those studies that do exist suffer from methodological issues that make general conclusions difficult to draw (Solon-Biet et al., 2014, Mitchell et al., 2015a, Speakman et al., 2016) see introduction above). We present an empirical study that directly tests the effect of dietary macronutrient content against calorie content in a vertebrate species and, critically, uses the GF and avoids the potentially confounding effect of dietary dilution (see Speakman et al., 2016). Overall we found that mortality risk and reproduction are determined by dietary macronutrient content not calorie intake. However, the effect of macronutrients on mortality risk is much clearer in males than in females. These results challenge the suggestion of fundamental differences in the mode of action of DR between vertebrate and invertebrate species (Speakman et al., 2016), and provide novel evidence of sex differences in the effect of macronutrients on mortality risk.

Our results show sex specific effects of macronutrient intake on lifespan but not reproduction. Male mortality risk was strongly affected by the lipid content of the diet, being lowest on more balanced protein : lipid intakes, and increasing as intakes diverged from this. However, we found no evidence of an effect of caloric intake on

male mortality risk, with reducing caloric intake often increasing mortality risk. These patterns fit well with previous findings in insects (e.g. Maklakov et al., 2008, Jensen et al., 2015) and one in mice (Solon-Biet et al., 2014), showing significant non-linear effects of non-protein dietary components on male lifespan and that male lifespan is maximised on low protein diets. Interestingly fat deposition increases with increasing lipid content of the diet in these fish (Chapter 3). This matches recent results in mice, where individuals fed a low protein : carbohydrate ratio diet had increased adiposity and increased survival (Solon-Biet et al., 2014). Such evidence challenges the suggestion of a link between a reduction in adiposity and an increase in lifespan under DR (Barzilai et al., 1998, Picard and Guarente, 2005, Muzumdar et al., 2008). It would be interesting to test whether other health and fitness related traits, such as physical performance, cognition, and the rate of ageing in these traits, respond in a similar manner. This would indicate that these low protein and intermediate lipid intakes improve overall health as well as reducing mortality risk.

In contrast to males, we found no effect of lipid intake on mortality risk in females and a weaker, variable effect of protein intake. In time period one, corresponding to early life over winter survival, mortality risk was reduced on intakes with a high protein : lipid ratio. However, this effect was reversed for the remainder of the experiment with high protein : lipid intakes increasing mortality risk. As with males dietary macronutrient ratio appeared to be more important than calorie content – although mortality risk decreased with decreasing intake of a particular diet in time period 1 (Fig 1C), this effect was much weaker than the change in mortality risk across diets. High protein : lipid intakes increasing mortality risk fits well with recent literature, which generally show higher mortality on high

protein : non-protein intakes (e.g. Lee et al., 2008, Maklakov et al., 2008, Carey et al., 2008, Fanson et al., 2009, Fanson et al., 2012, Solon-Biet et al., 2014, Jensen et al., 2015). However, the effect of protein intake on early life survival is in direct conflict with these results. One possible explanation for this difference could be in how the data were analysed. In previous studies, intakes were quantified over a time period where growth had ceased and intakes were stable (e.g. Solon-Biet et al., 2014). This period typically corresponds to an adolescent/adult period, rather than juvenile or early life, where growth has stopped. Therefore, it is possible that a beneficial early life effect of protein intake is being overlooked in these studies. Furthermore, in *D. melanogaster* egg to pupae survival was maximised on a high protein : carbohydrate ratio (1.5:1 (Rodrigues et al., 2015)), in contrast adult lifespan, which was maximised on low protein : carbohydrate intakes (1:16 (Lee et al., 2008)). By applying survival analyses that allow time varying covariates, we were able to detect this early life benefit of protein. It would be interesting to apply these analytical techniques in other species to see if or how the effect of protein changed across ontogeny.

Our finding of sex specific effects of macronutrient intake on mortality risk tallies with a recent study in crickets (Maklakov et al., 2008), but contrast with studies in flies (Jensen et al., 2015) and mice (Solon-Biet et al., 2014, Solon-Biet et al., 2015). In addition, the sex differences reported by Maklakov et al. (2008) were driven by slight differences mortality risk at very high carbohydrate intakes, with male risk increasing where-as female risk declined. In our study, there were more fundamental differences between the sexes, with male mortality being strongly affected by lipid intake while female mortality was affected by protein intake –

although this affect was variable across time. The majority of explanations for sex differences in the effect of diet on survival centre on differences in the reproductive roles of males and females and this seems likely in sticklebacks. Sticklebacks are sex role reversed, which we suggest results in female sticklebacks following a 'live fast, die young' strategy typically seen in males of other species. However it is unclear how this would generate sex-differences in mortality risk. Here, we expose females to a near complete range of reproductive costs, whereas typically the sex following this live fast die young strategy does not experience such a range of reproductive costs (e.g. male *D. melanogaster* in Jensen et al., 2015). We suggest that exposing females to near complete reproductive costs and males to very high reproductive costs in the present study, has accentuated the differences in the effect of macronutrients on mortality risk (see Moatt et al., 2016 and Chapter 2). However, more studies comparing the effect of diet on mortality risk are needed, particularly studies where both sexes are exposed to near complete reproductive costs.

In contrast to mortality macronutrient intake had similar effects on reproduction in both male and female reproduction, with protein intake having a positive effect on reproduction in both sexes, and intermediate lipid intake have a significant effect on female reproduction. These findings fit well with the majority of recent work which suggest high protein diets are beneficial for reproduction (Hunt et al., 2004, Lee et al., 2008, Maklakov et al., 2008, Fanson et al., 2009, Fanson et al., 2012, Solon-Biet et al., 2015, Jensen et al., 2015). However, in contrast to Maklakov et al. (2008) and Jensen et al. (2015), we do not detect major differences in the effect of macronutrient intake on reproductive output between the sexes. Despite there being a non-linear effect of lipid intake on female reproduction, that wasn't present

for males, this sex difference was not significant. Coupled with the effect of diet on mortality risk our results hint at a potential trade-off between reproduction and lifespan in both sexes. Reproduction appears to be maximised at high protein : lipid intakes in both sexes, whereas lifespan is maximised at a more balanced protein : lipid intake in males and generally at low protein : lipid intakes in females. These results fit well with those generally reported in the literature, with reproduction maximised at high protein : carbohydrate diets, lifespan maximised at low protein : carbohydrates and fitness maximised at an intermediate ratio (e.g. Hunt et al., 2004, Lee et al., 2008, Carey et al., 2008).

Interestingly the male reproductive traits measured here were energetically costly behavioural traits (e.g. courtship). It could have been predicted that these traits would respond positively to lipid intake, a calorie dense dietary component, rather than protein intake, which is typically used for more structural components. However, we do not see evidence of that here, with high protein intakes resulting in greater investment in courtship. Furthermore, there was no effect of protein intake on nest construction, which determined when courtship assessment was carried out, suggesting that high protein intakes result in fundamental changes in behaviour – increasing time spent courting. One possible explanation for this lack of a lipid effect could be linked to the experimental design. As discussed in Chapter 3, male sticklebacks in the wild are unlikely to be able to forage during the breeding season (Rohwer, 1978), hence the need for greater adiposity. Here, males did not have any food limitation throughout the breeding season. It is therefore possible that males were able to utilise these lipid stores as an energy source for reproduction. Thus no males, even those on the lowest lipid diets, were actually limited in lipid availability

during the breeding season meaning we did not detect an overall effect of lipid. Had males been restricted in food availability during the breeding season, we may have then detected an effect of lipid on male reproduction.

Finally, we show clear patterns of reproductive senescence in both sexes but no sex-specific effects of nutrition on senescence. Although protein intake reduced the rate of reproductive decline in females but not males, this sex difference was not significant. These results conflict with two recent studies exploring the impact of macronutrient intake on reproductive senescence. In *T. commulus* females show clear patterns of reproductive senescence while males do not, but nutrition alters reproductive aging in males but not in females (Maklakov et al., 2009). In *D. melanogaster* both sexes experience reproductive senescence, with protein intake increasing female reproductive decline and decreasing males (Jensen et al., 2015). In line with both of these studies, we found that females reproducing for longer in the breeding season produced larger egg clutches on the whole. Furthermore, given that females here may be following a live fast die young strategy, it may be more appropriate to compare the females in the present study to the males in the previous studies (Maklakov et al., 2009, Jensen et al., 2015). In this case, our findings support those of Maklakov et al. (2009) and Jensen et al. (2015), with protein having a beneficial effect on reproductive senescence. An interesting question is why we did not detect any sex specific effects of macronutrient intake on senescence, despite the independent sex analyses showing clear differences? One possibility is that protein is more beneficial to higher cost reproductive behaviours. As females were exposed to near complete reproductive costs the beneficial effect of protein was easily detected (see Moatt et al., 2016 and Chapter 2). However, males did not face potentially the

most costly aspect of reproduction, egg fanning. This could have resulted in the beneficial effect of protein on senescence being harder to detect in males, but being detectable enough in the combined analysis to result in no difference between the sexes.

In conclusion, we provide the first evidence that even in the absence of dietary dilution, macronutrients rather than caloric intake underpin changes in lifespan and reproduction in a vertebrate species. Furthermore we show clear sex-specific optima in the effect of macronutrient intake on mortality risk but not reproduction or senescence, with evidence that diet may underlie the trade-off between reproduction and lifespan in both sexes. In conflict with current theories, we provide evidence for a potential link between lipid intake, increased adiposity and a reduction in mortality risk, but only in males. More studies are required to test whether these effects are species specific or evolutionarily conserved. In particular, a wider range of vertebrate studies are needed to test the suggestion of fundamental differences in the mode of action of DR in vertebrates and invertebrates (Speakman et al., 2016).

Chapter 5

The effect of diet on growth, condition and swimming performance.

Data collection for this chapter was carried out by JPM, EH, FM, LJMM & MF

5.1 Abstract

Diet is an important determinant of organismal fitness, with recent work highlighting the role of macronutrient intake. However, this work has focused on the key life-history traits of reproduction and lifespan, with other fitness associated traits often neglected. Using the three-spine stickleback, we explore the link between dietary macronutrient content and key fitness associated traits: growth, condition and physical performance. We find significant differences in growth between the sexes, with females being both heavier and longer than males. However, there was no difference in the effect of macronutrient intake on growth, with diets containing a balance of both protein and lipid maximising growth. We also found significant differences between the sexes in body condition, a measure of overall fish health, with males being in better condition than females. In contrast to growth, we found significant differences in the effect of macronutrients on condition with lipid intake improving condition more in males than females, but the opposite being true for protein intake. We found significant sex differences in swimming performance, but no effect of macronutrient intake in either sex. Our findings add weight to previous suggestions that the intake of no one macronutrient can maximise growth, rather diets balanced in numerous key macronutrients are required for growth to be maximised. The sex-specific effect of macronutrient intake on condition suggests that males and females utilise their ingested macronutrients differently, with lipid improving male condition more than females. Interestingly, it has previously been reported that male three spine-sticklebacks have higher adiposity and lower mortality risk with high lipid intakes, suggesting a link between fat storage, health and survival in male sticklebacks.

5.2 Introduction

Key fitness related traits such as reproduction and lifespan are influenced by diet and nutrition (Partridge et al., 2005, Fontana and Partridge, 2015). Dietary restriction (DR), a reduction in the intake of calories or specific macronutrients, extends lifespan and protects against age related diseases (reviewed Nakagawa et al., 2012, Selman, 2014). Originally thought to act through a reduction in calories (Mccay et al., 1935; reviewed Speakman and Mitchell, 2011), recent studies employing a powerful integrative approach, the geometric framework of nutrition (GF), suggest that variation in the ratio of protein : non-protein energy in the diet is the key component linking diet and lifespan (reviewed Simpson et al., 2017). Despite much interest in applying the GF to DR studies, these typically focus on the key life-history traits of reproduction and lifespan (e.g. Carey et al., 2008, Lee et al., 2008, Maklakov et al., 2008, Fanson et al., 2009, Jensen et al., 2015). Few studies have attempted to apply the GF to study other key fitness related traits such as growth, body condition and physical performance, which may be important in linking diet and lifespan. Here, through use of the GF and employing a fresh water fish, the Three-spine Stickleback (*Gasterosteus aculeatus*), as our model we investigate the relationship between macronutrient intake, growth and performance.

Early work on DR in rats (*Rattus norvegicus*) recognised the potential importance of growth as a trait that may link diet and lifespan, suggesting that it was through retardation of growth that lifespan was extended under restricted diets (Osborne et al., 1917, Mccay et al., 1935). This reduction in growth was achieved through caloric restriction (CR), restricting the overall calorie intake of individuals (Mccay et al., 1935). Consequently, it is well known that CR reduces bodyweight

(e.g. Colman et al., 1998; reviewed Speakman and Mitchell, 2011). However, less is known regarding the link between macronutrient intake and growth and how this may link to lifespan and reproduction. In the fruit fly, *Drosophila melanogaster*, body weight increases with increasing ratio of protein : carbohydrate, however the pattern of change was biphasic, with change in body weight occurring much slower as protein : carbohydrate ratio increased from 1:2 to 4:1 than over lower protein : carbohydrate ratios (Lee, 2015). Interestingly, this study found that lifespan was maximised at two intermediate protein : carbohydrate intakes of 1:2 and 1:4 (Lee, 2015), suggesting that diets maximising lifespan do not minimise growth. In mice, *Mus musculus*, it has been repeatedly shown that growth and body weight are maximised on balanced diets, typically on a protein : carbohydrate ratio of around 1:2 (Sørensen et al., 2008, Huang et al., 2013, Solon-Biet et al., 2014). As the ratio of protein : carbohydrate diverges from this peak, body weight decreases. Interestingly, in mice, increases in adiposity were greatest on low protein : carbohydrate diets, but these diets also had the lowest lean mass growth, resulting in a smaller overall body weight (Sørensen et al., 2008, Huang et al., 2013, Solon-Biet et al., 2014). Paradoxically, given the well-known association between increased adiposity and a reduction in lifespan (Barzilai et al., 1998, Picard and Guarente, 2005, Muzumdar et al., 2008), mice on these low protein : carbohydrate diets had the highest survival, despite their increase in fat deposition (Solon-Biet et al., 2014).

There is also a wide body of evidence from the fields of agriculture and aquaculture on the impact of diet on growth. Although these studies generally focus on the low cost production of meat products for human consumption, they often explore the effect of varying macronutrient intake on growth. In chickens,

Gallus gallus domesticus, increasing protein content of the diet resulted in an increase in overall weight (Donaldson et al., 1956, Aletor et al., 2000), with greater food consumption required on low protein diets to achieve the same weight as high protein diets (Aletor et al., 2000). In European Whitefish, *Coregonus lavaretus*, weight increased with increasing protein content of the diet, peaking at a balanced diet with a protein : lipid ratio of 2.25:1 (Ruohonen et al., 2003, Ruohonen et al., 2007). A similar pattern was also seen in red tilapia, with increasing growth with increasing ratio of protein : lipid (De Silva et al., 1991). Therefore the general pattern is that growth increases with increasing protein content of the diet, with the a potential peak at diets with a balance of protein : non-protein energy.

In Chapter 3, we showed that stickleback body weight was highest on a diet with a protein : lipid ratio of *1:2.5*, with no other differences in weight on the remaining four diets. We suggested that this was because a balance between protein, lipid and energy density was required to maximise growth (see Chapter 3). We also showed that carcass protein and fat content were determined by lipid intake, not protein intake, with increasing lipid intake reducing protein and increasing lipid content of the carcass (see Chapter 3). However in Chapter 3, sticklebacks were fed five diets with varying protein : lipid ratio *ad libitum*, with no assessment of the effect of overall caloric intake. Furthermore, we did not have information on an individual's specific intake of macronutrients (e.g. protein intake $gday^{-1}$), rather we looked at the effect of the percentage of protein and lipid in the diet. Thus we did not have the information to apply a full GF type analysis to this growth data.

In ecological studies, a common method for assessing an individual's overall health is through condition analysis, or comparing their relative physical condition to that of the population. Assessment of condition is used in many species to indicate health (e.g. deer (Kie et al., 1983), birds (Carrascal et al., 1998, Gosler and Harper, 2000) and fish (Bentley and Schindler, 2013; and Chapter 3), with methods for quantifying condition often being relatively species specific. For example, in deer common measures of assessing condition are: subcutaneous fat (e.g. back fat), visceral fat (e.g. kidney fat) and blood serum lipid concentration (Kie et al., 1983). Whereas in birds common condition measures are pectoral muscle thickness (Carrascal et al., 1998, Gosler and Harper, 2000) and fat stores (Gosler and Harper, 2000). In fish there is a relatively well defined method of assessing overall condition, by comparing the actual weight of a fish to the predicted weight for a fish of the same size (see Materials and Methods below). In Chapter 3 we showed that fish on a protein : lipid ration of *4.6:1* were in significantly poorer body condition than fish on *8.5:1* and *2.5:1* diets. However, these individuals were kept on diet treatments for a relatively short period of time, meaning we could not assess the effect of long term diet treatment on condition. Furthermore, as discussed previously, these fish were fed *ad lib* with no caloric restriction or quantification of intake, meaning we were unable to analyse the effect of specific macronutrient intake on condition. Thus the current study offers an exciting opportunity to explore how specific macronutrient and calorie intake affects body condition, and thus overall health.

Physical performance, such as endurance tasks and activity, are well known to be influenced by CR (reviewed Speakman and Mitchell, 2011), with endurance tasks suggested to be indicators of underlying physical condition and, therefore,

health. When subjected to CR, mice have improved rotorod performance (ability to remain on a plastic rod rotating at 3rpm, (Ingram et al., 1987)), higher run-wheel performance (Ingram et al., 1987) and improved hang time (ability to remain hanging from a suspended wire (Means et al., 1993)). However, to date there is little to no indication of how varying macronutrient intake will effect endurance. Recent evidence suggests that CR rather than protein restriction drives changes in activity, but the effect of CR varied depending on the measure of activity being used (Mitchell et al., 2016). However, Mitchell et al. (2016) did not measure any physical endurance trait. In Chapter 3, we showed there was no effect of dietary protein : lipid ratio on physical performance. However, as with weight (discussed above), fish were fed the five diets *ad lib* with no quantification of intake. Therefore, we could not utilise the GF to explore how macronutrient or calorie intake effects performance.

Here by using three-spined sticklebacks reared on 15 diets with varying protein, lipid and calorie intakes, we use the GF to address the following questions: (1) What is the effect of macronutrient and caloric intake on growth and condition? (2) What is the effect of macronutrient and caloric intake on physical performance? (3) Are the sex differences in the effect of macronutrients on these traits? To distinguish between the effects of macronutrient and calorie intake we use the GF (summarised Simpson and Raubenheimer, 2012 and See Chapter 4). By providing multiple diets with varying macronutrient contents, at multiple restriction levels, it is possible to plot response of key fitness related traits, such as growth, onto this nutrient space (Simpson and Raubenheimer, 2012). Given the results presented in Chapter 3, we predict that growth will be highest on the diet with the best balance, containing both high protein and lipid contents as well as a high energy density.

Furthermore, we predict that performance will be higher on the diets with high lipid contents, as these diets have high energy densities. Finally, given the results of Chapter 3, we predict that there will be significant sex differences in growth, with females being larger than males. However, we do not expect to see any sex differences in swimming endurance.

5.3 Materials and Methods

5.3.1 Husbandry

Experimental individuals were the fish used in the study of reproduction and mortality reported in Chapter 4. Briefly, fish were first generation offspring of wild caught three-spine sticklebacks. Fish were maintained on a diet of live artemia and fry powder (ZM Systems, ZM-100 Fry Food: protein 55.0%, oil 13.0% and ash 12.0%) until three months of age, and from three to four months of age they were fed standard grade fish pellet (ZM Systems, medium granular: protein 52.0%, oil 12.0% and ash 10.3%) to condition them to surface feeding. Fish were molecularly sexed through fin clips at 4 months of age and assigned to one of 15 diet treatments (n= 20 per sex, per dietary treatment, total n=600, see Appendix 3: Table S1). Fish were housed in flow through glass sided aquariums split into (7 x 25 x 50cm) compartments housing a single fish and containing an artificial plant. Temperature and light regimes were matched to the natural levels for Edinburgh at that time of year. Clutches and treatments were evenly distributed between stacks and shelves to control for both family and tank effects.

5.3.2 Dietary treatments

Diets were the same as those reported in both chapter 2 and 3 (see Appendix 3: Table S3.2). In brief, a total of five diets were used which varied in the ratio of protein to lipid (Table 5.1) and containing indigestible carbohydrate filler (Kim and Kaushik, 1992, Guillaume, 2001). Diets were provided at three levels; 100% (*ad libitum*), 75% and 50% of *ad lib*, giving a total of 15 dietary treatments. To avoid problems associated with dietary dilution (see Chapter 4), we used an intermittent feeding regime with individuals in the 100% treatment fed twice a day, the 75% treatment fed alternately once a day and then twice on the second day and the 50% treatment were fed once a day. Feeding levels were quantified using monthly monitoring of sentinel fish for each diet from the 100% treatment (See Appendix 3). Within each treatment, fish were classified as either large (heaviest 10 fish) or small (lightest 10 fish) for each sex, to account for differences in feeding rate between fish of different size. This resulted in 60 different feeding quantities (sex*diet*level*size combination) each being fed to 10 individuals (see Appendix 3: Table S3.1).

Table 5.1 Table of the nutrient content of the five diets used in this experiment. Note, carbohydrate in these diets is indigestible filler.

Protein (%)	Lipid (%)	Carbohydrate (%)	Ratio P:L	Calories (MJ/kg)
67.5	6.6	15.8	10.2 : 1	19.3
33.2	3.9	53.1	8.5 : 1	17.5
59.3	13.0	16.1	4.6 : 1	20.2
51.6	20.5	17.8	2.5 : 1	22.2
31.2	19.2	39.7	1.6 : 1	21.5

5.3.3 Growth

At four months of age, prior to the start of dietary manipulations, fish were weighed (g) and length taken (mm). We used a standard weighing procedure where fish are removed from their tank, dried and weighed on a balance (± 0.01g). Once weighed the length (± 0.5mm) of the fish was measured from head to tail fork and the fish was returned to its original tank, with the whole process taking less than 60s. Fish were weighed approximately every 1-2 months from the start of the experiment (November 2014) until the end (December, 2016; Table 5.2). Partial data for the August 2015 weighing was lost, however this was a period of little to no growth. For the analysis presented below, we only look at growth over the first 10 measurements (November 2014 – May 2016), as from July 2016 onwards there were few individuals alive, resulting in lack of statistical power (see Table 5.2).

5.3.4 Condition index

A common measure of assessing overall health of a fish is condition index, a measure of the weight of an individual relative to its size (length). Here, we calculated condition using residuals from an analysis of the length-weight relationship (see Bentley and Schindler, 2013 and Chapter 3):

$$\text{Condition Index} = \log(Weight) - \log(a) - b\log(Length)$$

With the slope (b) and intercept (a) taken from a model of the log of weight against the log of length for all fish measured in this study (Bentley and Schindler, 2013). A negative value indicates an individual weighing less than average for its length, whilst a positive value suggests an individual weighing more than average for its length. Condition index was calculated for each batch independently, meaning a

value of 0 is the average condition for each batch. Both sexes were included in the intercept and slope calculation, making the condition index score relative to the whole population.

Table 5.2 Table showing the number of individuals alive at each weighing batch, for each sex. Some data was lost for the August 2015 weighing.

	Diet (p:l)									
	10.2:1		**8.5:1**		**4.6:1**		**2.5:1**		**1.6:1**	
Time	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Nov 2014	61	59	60	59	60	60	57	63	61	59
Dec 2014	54	56	56	58	53	59	56	60	60	55
Jan 2015	43	55	52	57	51	57	54	58	54	51
Mar 2015	38	52	51	52	47	55	51	57	53	47
June 2015	35	46	48	47	47	53	50	50	51	47
Aug 2015	*13*	*17*	*22*	*20*	*20*	*25*	*14*	*23*	*27*	*25*
Oct 2015	18	22	35	30	31	27	40	25	41	27
Dec 2015	16	19	32	26	31	26	39	20	39	22
Feb 2016	16	16	31	20	27	24	38	18	37	19
May 2016	14	13	27	15	26	20	36	15	35	16
Jul 2016	11	6	18	11	20	9	27	7	27	8
Sep 2016	9	4	11	7	17	3	14	4	15	2
Nov 2016	8	2	8	1	13	1	10	3	11	1
Dec 2016	8	2	8	1	10	1	9	2	11	1

5.3.5 Swimming endurance

Fish were assessed for their swimming endurance ability using a similar protocol as described in Chapter 3 (also see Alvarez and Metcalfe, 2005). Briefly, fish were placed in a swim chamber (length 25cm, internal diameter 6cm) submerged in a glass sided tank (59 x 29 x 28cm) filled to a depth of 22cm with room temperature water. Within the swim chamber, fish were exposed to two currents, initially a slow current (4cms^{-1}) for 5 minutes, to condition individuals to the swim chamber, after which the speed was increased to 20cms^{-1} and a timer started. At the

first cessation of swimming, fish were prompted to return to swimming by a small tap on the chamber. If this failed to elicit swimming, or at the second refusal to swim, the current and timer were stopped. Where individuals continued to swim, the trial was allowed to run for a maximum of 20 minutes (5 minutes acclimatisation and 15 minutes at $20cms^{-1}$). Immediately following the trail, the fish was removed to a recovery tank and a 50% water change performed before another trial was initiated. Temperature was recorded every two hours, then converted into a daily average. Swimming endurance was taken as the time an individual was able to remain swimming while exposed to the high speed current and any fish that swam for the full trial was given a score of 15 minutes (trial 1: n=19 out of 507 tested; trial 2: n=0 out of 202 tested). Fish were tested twice in their lifetime, with trial 1 being performed at the start of the breeding season (mean age ± s.e. = 126.50 ± 0.90; n = 241 males, 265 females) and trial 2 performed after the breeding season (mean age ± s.e. = 312.47 ± 0.33; n = 168 males, 139 females). These time points were picked as the breeding season represents a period of significant investment for sticklebacks, which were likely to impose significant costs on performance.

5.3.6 Statistical analysis

All analyses were carried out in R (v3.4.0, R core team, 2017). We used a multivariate response-surface approach (Lande and Arnold, 1983) to estimate the linear and non-linear effects (quadratic and correlation) of protein and lipid intake and the interaction between them on our response variables (e.g. Lee et al., 2008, Maklakov et al., 2008, Maklakov et al., 2009, Fanson et al., 2009, Solon-Biet et al., 2014, Jensen et al., 2015 and see below). As recommended (Lande and Arnold, 1983) estimates of linear terms were taken from a model only containing linear terms

whereas estimates of non-linear terms were taken from a model including linear and non-linear terms. For all analyses, protein and lipid intakes were standardised to a mean of zero and a standard deviation of one to avoid issues of scale differences when fitting quadratic terms. For all traits we performed separate analyses for each sex to test for sex specific effects of macronutrients. We then combined the data and performed a full analysis with sex interacted with protein and lipid to test if the effect of macronutrients differed between the sexes. Nutritional landscapes were visualised using thin-plate splines from the package *fields* (Nychka et al., 2016).

Weight, length and condition index were analysed through general linear mixed model using ASReml-R software (v3.0, Gilmour et al., 2009). For initial differences across treatments, response variables were modelled against Diet and Restriction Level (treatment) fitted as factors. To test for initial differences between the sexes, a separate analysis was performed with sex included as factor. When testing the effect of macronutrient intake, protein and lipid intakes were calculated as the average daily intake ($gday^{-1}$) for the period between each measurement (i.e. the average daily intake for the period from batch 1 to batch 2), with the linear and non-linear effects of protein and lipid included as continuous covariates. All models included batch as a factor, with protein and lipid being interacted with batch to test for changing effects over time. For models exploring differences between the sexes, sex was included as a factor and then interacted with each variable. If there was evidence of an interaction between sex and a specific dietary component, the three way interaction between batch, sex and the macronutrient was included to check for changing effects over time. Were included a first order autoregressive function on the residual covariance matrix to allow different residual variance at each batch.

Swimming endurance, time spent swimming at the high current, was analysed via a Markov Chain Monte Carlo generalised linear mixed model (MCMCglmm) (Hadfield, 2010) using a Poisson distribution, due to a number of fish failing to swim. To minimize autocorrelation between successive samples of the model it was run for 1,300,000 iterations and a burnin of 300,000 with 1000 samples stored, with the exception of the model looking at sex differences in trial 2 swimming performance, where it was necessary to run the model for 1,950,000 iterations. The linear and non-linear effects of protein and lipid, their interaction, fish weight and water temperature were included as continuous covariates, with fish clutch included as a random effect. The change in swim time (difference in high current swim time between trial 1 and trial 2) was analysed through linear mixed effects models (LMER) using the package *Lme4* (Bates et al., 2015) and included the same fixed effects detailed previously. A separate analysis was carried out for each sex and trial, then to test for sex differences, a single model for each trial was performed with sex included as a factor.

5.4 Results

5.4.1 Weight and length

Males

For males, there were no differences between treatments in initial weight (LME; Diet: $\chi^2 = 0.21$; $p = 0.647$; Level: $\chi^2 < 0.00$; $p = 0.973$; Appendix 4: Fig. S4.1). There was a significant effect of batch on male weight, with weight increasing with time (Wald test: $F_{8, 763.6} = 404.50$; $p < 0.001$; Fig 5.1; Appendix 4: Table S4.1 & Fig. S4.1). There was a significant non-linear effect of protein on male weight, with

weight being greatest on intermediate protein intakes (Wald test; Protein^2: $F_{1, 1364.8} = 17.88$; $p < 0.001$; Protein: $F_{1, 824.5} = 7.99$; $p = 0.005$; Fig 5.1; Appendix 4: Table S4.1). However, there was a significant interaction between the non-linear effect of protein and batch (Wald test; Batch*Protein^2: $F_{8, 848.1} = 2.41$; $p = 0.014$; Batch*Protein; $F_{8, 860.7} = 0.65$; $p = 0.739$; Fig 5.1; Appendix 4: Table S4.2), this suggests the non-linear effect of protein remains becomes significantly more curved at final weighing (see Appendix 4: Table S4.2). As with protein, there was a significant non-linear effect of lipid intake on weight, again with weight being highest on intermediate lipid intakes (Wald test; Lipid^2: $F_{1, 1193} = 17.39$; $p < 0.001$; Lipid: $F_{1, 667.2} = 10.68$; $p = 0.001$; Fig 5.1; Appendix 4: Table S4.1). As with protein, the non-linear effect of lipid changed over time, becoming significantly more curved at final weight batch (Wald test; Batch*Lipid^2: $F_{8, 837.3} = 5.19$; $p < 0.001$; Batch*Lipid; $F_{8, 832.2} = 7.73$; $p < 0.001$; Fig. 5.1; Appendix 4: Table S4.2). Finally, there was a significant interaction between protein and lipid, with the effect of lipid being greater at high protein intakes and vice versa (Wald; $F_{1, 1128.7} = 21.52$; $p < 0.001$; Appendix 4: Table S4.1), which changed over time with the interaction strongest in batch 3, before declining at later batches (Wald; Batch*Protein*Lipid: $F_{8, 821} = 2.07$; $p = 0.036$; Appendix 4: Table S4.3).

The overall effects of time and macronutrients on male length were the same as those for male weight (see Appendix 4: Table S4.3), with there being significant effects of batch ($p < 0.001$), protein^2 ($p = 0.022$), lipid^2 ($p < 0.001$) and an interaction between protein and lipid ($p = 0.004$; Fig. 5.2; Appendix 4: Table S4.3 & Fig. S4.2). However, the interactions between macronutrients and batch were different for length than for weight. There was significant interaction between batch and lipid for

length (Wald test; Batch*Lipid: $F_{8, 820.7} = 3.68$; $p < 0.001$; Fig. 5.2; Appendix 4: Table S4.4), with the positive effect of lipid intake on length getting stronger over time.

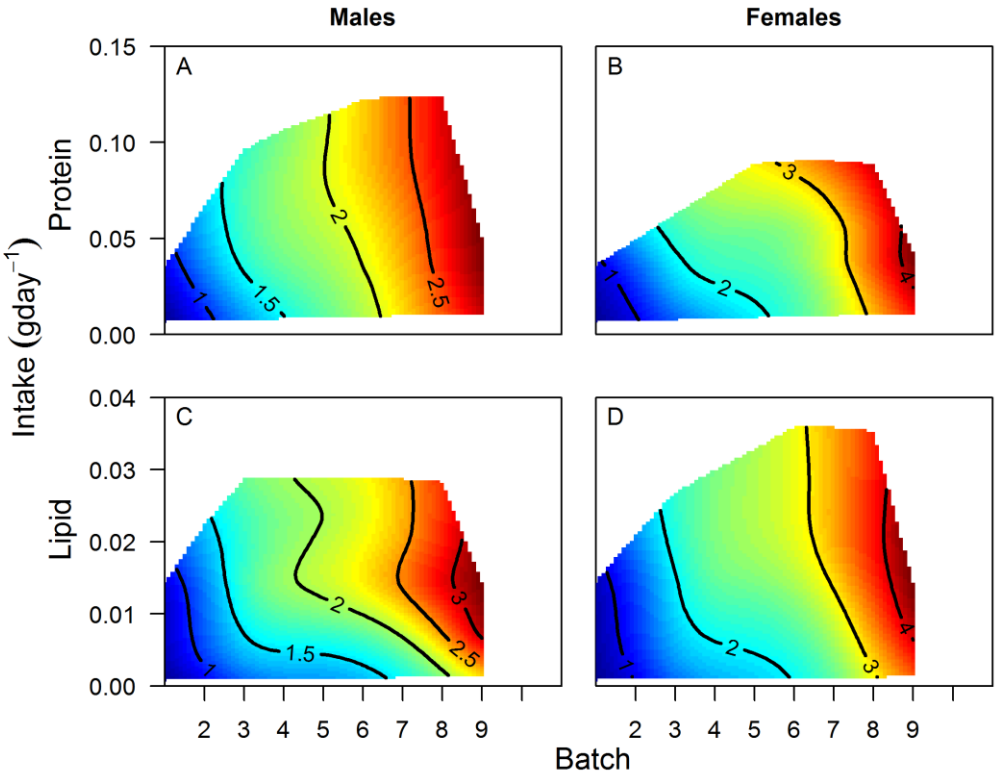

Figure 5.1 Non-parametric thin-plate spline contour visualisations for the effect of protein and lipid intake (gday^{-1}) on weight (g) across batches. Panel response surfaces as follows: (A) effect of protein on male weight, (B) effect of protein on female weight, (C) effect of lipid on male weight, and (D) effect of lipid on female weight. There were no differences in the effect of macronutrients between the sexes ($p < 0.2$).

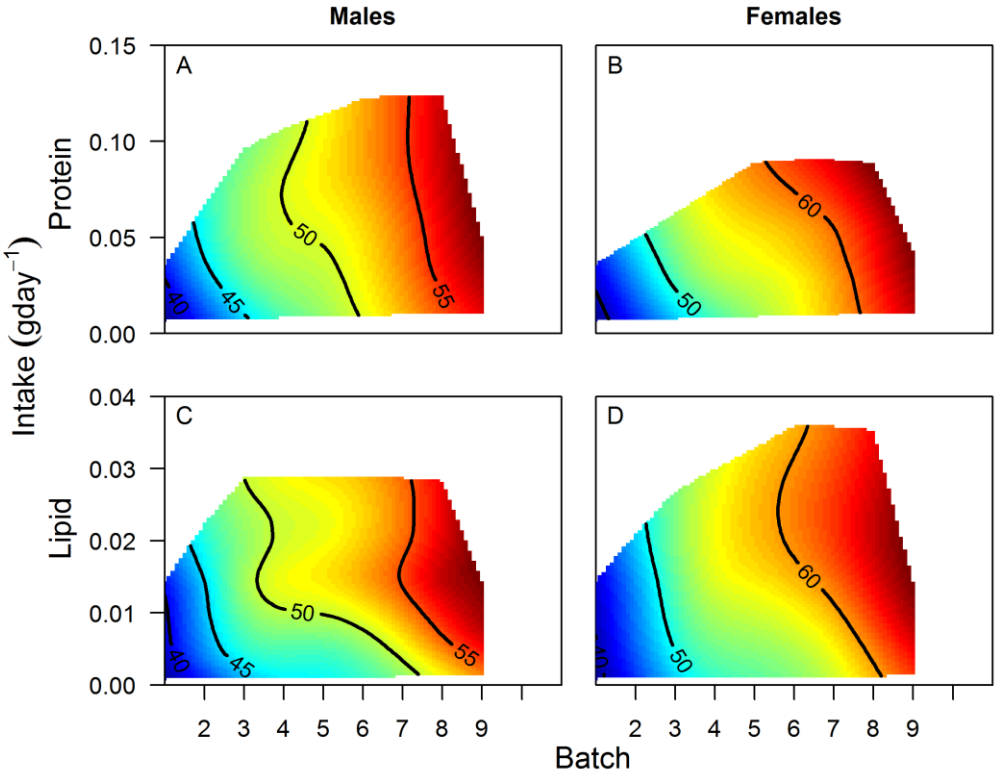

Figure 5.2 Non-parametric thin-plate spline contour visualisations for the effect of protein and lipid intake (gday^{-1}) on length (mm) across batches. Panel response surfaces as follows: (A) effect of protein on male length, (B) effect of protein on female length, (C) effect of lipid on male length, and (D) effect of lipid on female length. There were no differences in the effect of macronutrients between the sexes (all $p < 0.2$).

Females

For females, as with males, there were no differences in initial weight between treatments (LME; Diet: $\chi^2 = 0.02$; $p = 0.895$; Level: $\chi^2 = 0.05$; $p = 0.976$; Appendix 4: Fig S4.1). Similar to males, weight increased with increasing batch (Wald test; $F_{8, 564.4} = 319.2$; $p < 0.001$; Fig. 5.1; Appendix 4: Table S4.5 & Fig. S4.1). Unlike males, there was no significant effect of protein on weight (Wald test; Protein: $F_{1, 756.5} = 2.84$; $p = 0.093$; Protein^2: $F_{1, 647.2} = 0.75$; $p = 0.651$; Fig 5.1;

Appendix 4: Table S4.5). However, there was a significant non-linear effect of lipid, with intermediate intakes maximising growth (Wald test; Lipid^2: $F_{1, 637.2} = 2.43$; $p = 0.014$; Lipid: $F_{1, 567.1} = 1.78$; $p = 0.183$; Fig 5.1; Appendix 4: Table S4.5), which changed over time, with the effect of lipid getting more curved with increasing time (Wald test; Batch*Lipid^2: $F_{8, 633.2} = 1.59$; $p = 0.126$; Batch*Lipid; $F_{8, 597.7} = 3.66$; $p < 0.001$; Fig. 5.1; Appendix 4: Table S4.6). Unlike in males, there was no evidence of an interaction between protein and lipid (Wald test; $F_{1, 642.8} = 1.18$; $p = 0.306$; Appendix 4: Table S4.5).

As with males, the overall effects were the same for length as for weight (see Fig. 5.2, Appendix 4: Table S4.7), with significant effects of batch ($p < 0.001$) and lipid^2 ($p < 0.001$). However, in contrast to weight the interaction between protein and lipid had a significant effect on length (Wald test; Protein*Lipid: $F_{1, 701.0} = 5.78$; $p = 0.016$; Appendix 4: Table S4.7). Also in contrast with weight, the interaction between batch and lipid has a significant effect on length (Wald; Batch*Lipid: $F_{8, 609.2} = 3.15$; $p = 0.002$; Fig 5.2; Appendix 4: Table S4.8), suggesting the positive effect of lipid on length increased in strength across batches.

Comparing the sexes

There were significant differences between the sexes in initial weight (LME; Weight: $\chi^2 = 14.86$; $p < 0.001$; Fig. 5.3) and initial length (LME; Length: $\chi^2 = 25.04$; $p < 0.001$; Fig. 5.3), with females being heavier (mean weight (g) ± s.e.: males = 0.46 ± 0.01; females = 0.50 ± 0.01; Fig. 5.3) and longer than males (mean length (mm) ± s.e.: males = 34.09 ± 0.24; females = 35.50 ± 0.22; Fig. 5.3). This difference remained throughout the course of the experiment and increased over time for both

weight (Wald test; Sex: $F_{1, 778.3}$= 0.10; $p < 0.001$; Sex*Batch: $F_{8, 1235.8}$= 37.86; $p < 0.001$; Fig. 5.3; Table 5.3) and length (Wald test; Sex: $F_{1, 752.9}$ = 229.90; $p < 0.001$; Batch*Sex: $F_{8,}$ 1448.4= 4.50; $p < 0.001$; Fig. 5.3; Table 5.4). However, there was no difference between the sexes in the effect of macronutrient intake on weight (all $p > 0.3$; Fig. 5.1; Table 5.3) or length (all $p > 0.2$; Fig. 5.2; Table 5.4).

Table 5.3 Outputs from model of the sex differences in the effect macronutrient intake on weight. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test. For sex comparisons females are the reference level.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	0.692 (0.031)	2778	1, 1475.3	< 0.001
Batch 2	0 (NA)			
Batch 3	0.333 (0.017)			
Batch 4	1.088 (0.027)			
Batch 5	1.335 (0.029)			
Batch 6	1.414 (0.038)			
Batch 7	1.584 (0.039)			
Batch 8	1.905 (0.046)			
Batch 9	2.594 (0.06)			
Batch 10	3.084 (0.116)	757.4	8, 1340.9	< 0.001
Protein	0.222 (0.053)	0.323	1, 1511	0.570
Lipid	0.213 (0.071)	19.55	1, 1498.7	< 0.001
Protein^2	-0.287 (0.063)	20.39	1, 2208.4	< 0.001
Lipid^2	-0.169 (0.105)	35.16	1, 2112	< 0.001
Protein*Lipid	0.134 (0.028)	22.04	1, 1775.1	< 0.001
Sex (Male)	-0.028 (0.02)	0.10	1, 778.3	< 0.001
Batch 2*Lipid	0 (NA)			
Batch 3*Lipid	0.216 (0.035)			
Batch 4*Lipid	0.317 (0.051)			
Batch 5*Lipid	0.454 (0.056)			
Batch 6*Lipid	0.445 (0.079)			
Batch 7*Lipid	0.498 (0.074)			

Table 5.3 continued.

	Estimate (± s.e.)	F	d.f. (1,2)	*p*
Batch 8*Lipid	0.556 (0.088)			
Batch 9*Lipid	0.687 (0.112)			
Batch 10*Lipid	1.569 (0.255)	11.31	8, 1455.4	< 0.001
Batch 2*Lipid^2	0 (NA)			
Batch 3*Lipid^2	-0.257 (0.066)			
Batch 4*Lipid^2	-0.3 (0.078)			
Batch 5*Lipid^2	-0.377 (0.083)			
Batch 6*Lipid^2	-0.373 (0.098)			
Batch 7*Lipid^2	-0.403 (0.094)			
Batch 8*Lipid^2	-0.436 (0.103)			
Batch 9*Lipid^2	-0.503 (0.118)			
Batch 10*Lipid^2	-1.939 (0.467)	4.13	8, 1521.8	< 0.001
Batch 2*Sex (m)	0 (NA)			
Batch 3*Sex (m)	-0.031 (0.015)			
Batch 4*Sex (m)	-0.27 (0.031)			
Batch 5*Sex (m)	-0.413 (0.033)			
Batch 6*Sex (m)	-0.378 (0.043)			
Batch 7*Sex (m)	-0.442 (0.042)			
Batch 8*Sex (m)	-0.582 (0.052)			
Batch 9*Sex (m)	-0.86 (0.071)			
Batch 10*Sex (m)	-1.169 (0.087)	37.86	8, 1235.8	< 0.001

Table 5.4 Outputs from model of the sex differences in the effect macronutrient intake on length. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test. For sex comparisons females are the reference level.

	Estimate (± s.e.)	F	d.f. (1,2)	*p*
(Intercept)	38.81 (0.464)	48830.00	1, 1132.9	< 0.001
Batch 2	0 (NA)			
Batch 3	5.605 (0.267)			
Batch 4	12.023 (0.352)			
Batch 5	15.368 (0.390)			
Batch 6	15.723 (0.449)			
Batch 7	17.564 (0.541)			
Batch 8	18.696 (0.549)			

Table 5.4 continued

	Estimate (± s.e.)	F	d.f. (1,2)	*p*
Batch 9	24.164 (0.611)			
Batch 10	27.494 (0.681)	728.00	8, 1474.5	< 0.001
Protein	0.781 (0.682)	0.06	1, 2351.9	0.806
Lipid	3.143 (0.649)	63.79	1, 1500.3	< 0.001
Protein^2	-1.492 (0.820)	3.309	1, 1598.3	0.069
Lipid^2	-4.030 (0.705)	32.66	1, 1554.7	< 0.001
Sex (male)	-0.988 (0.358)	229.90	1, 752.9	< 0.001
Protein*Lipid	0.628 (0.279)	5.08	1, 1493.4	0.024
Batch 2*Protein	0 (NA)			
Batch 3*Protein	0.388 (0.332)			
Batch 4*Protein	-0.132 (0.430)			
Batch 5*Protein	0.253 (0.480)			
Batch 6*Protein	0.390 (0.531)			
Batch 7*Protein	0.452 (0.581)			
Batch 8*Protein	0.470 (0.587)			
Batch 9*Protein	0.465 (0.609)			
Batch 10*Protein	0.589 (0.754)	35.65	8, 1343.7	< 0.001
Batch 2*Lipid	0 (NA)			
Batch 3*Lipid	1.009 (0.243)			
Batch 4*Lipid	1.653 (0.334)			
Batch 5*Lipid	1.952 (0.374)			
Batch 6*Lipid	2.160 (0.417)			
Batch 7*Lipid	2.150 (0.463)			
Batch 8*Lipid	1.904 (0.471)			
Batch 9*Lipid	2.366 (0.504)			
Batch 10*Lipid	2.587 (0.668)	1.52	8, 1492.9	0.144
Batch 2*Sex (m)	0 (NA)			
Batch 3*Sex (m)	-0.411 (0.233)			
Batch 4*Sex (m)	-2.345 (0.311)			
Batch 5*Sex (m)	-4.556 (0.347)			
Batch 6*Sex (m)	-5.417 (0.442)			
Batch 7*Sex (m)	-5.504 (0.567)			
Batch 8*Sex (m)	-5.065 (0.578)			
Batch 9*Sex (m)	-6.353 (0.669)			
Batch 10*Sex (m)	-8.072 (0.648)	4.50	8, 1448.4	< 0.001

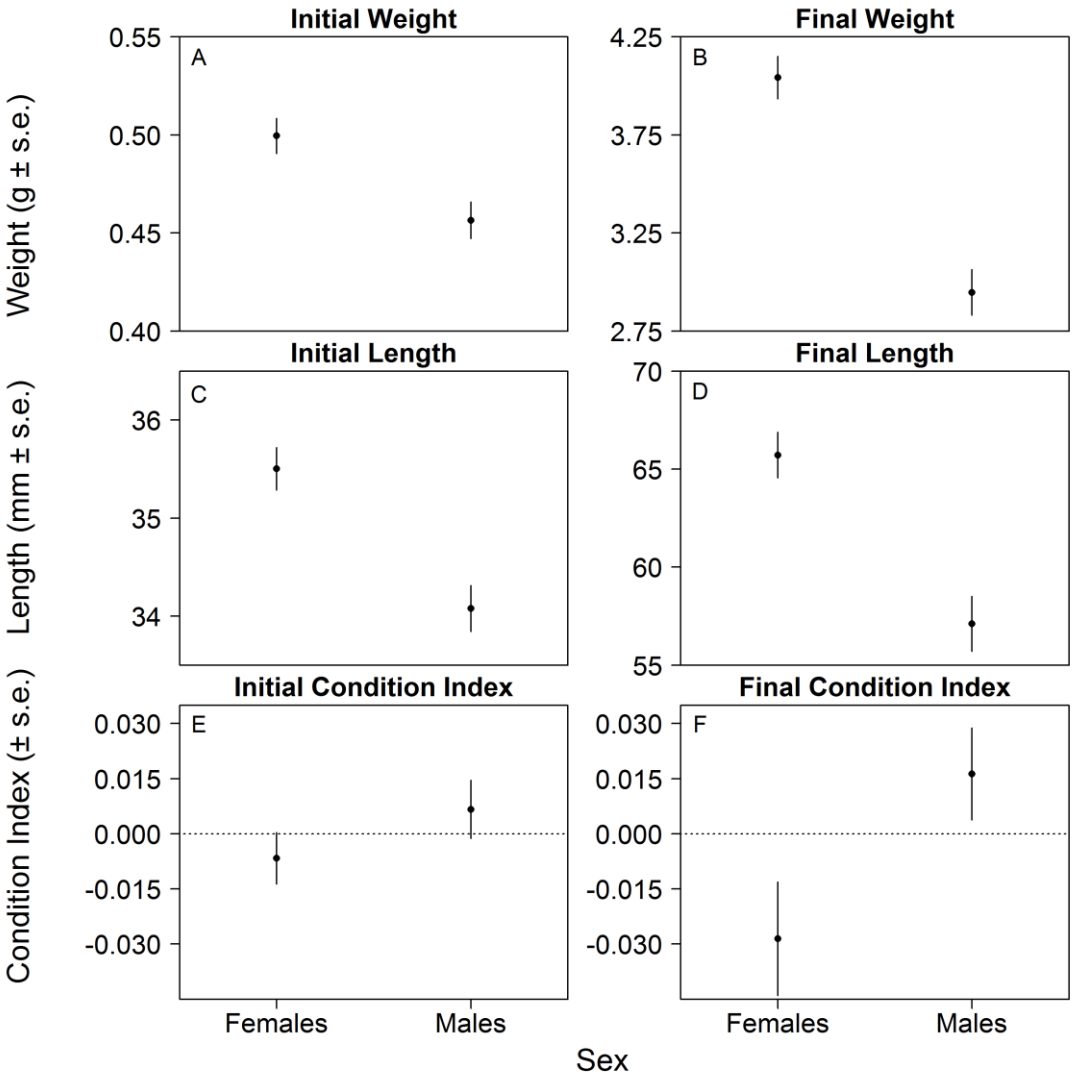

Figure 5.3 Sex differences in growth and condition. Values represent the mean (± s.e.) for each sex. Panels as follows: (A) initial weight, (B) final weight, (C) initial length, (D) final length, (E) initial condition index and (F) final condition index. For condition index, zero is average condition (dashed line), with a positive value indicating a better than average condition, a negative value worse than average. There were significant differences in initial weight and length ($p < 0.001$) and these differences increased through the course of the experiment ($p < 0.001$). There was no difference in initial condition ($p = 0.197$) but at final measurement females were in significantly worse condition than males ($p < 0.001$).

5.4.2 Condition index

Males

There were no initial differences in male condition across treatments (LME; Diet: $\chi^2 = 1.06$; $p = 0.303$; Level: $\chi^2 = 0.04$; $p = 0.842$; Appendix 4: Fig.S4.3). Male condition differed significantly across batches (Wald test; $F_{8, 678.7} = 11.56$; $p < 0.001$; Fig. 5.4; Appendix 4: Table S4.9 & Fig. S4.3). However, unlike length and weight, condition did not increase linearly across batches, rather visual inspection suggests there was an initial increase in condition, followed by a sharp decline around batch 7 (see Fig. 5.4 and Appendix 4: Fig. S3). There was a positive linear effect of lipid intake on male condition (Wald test; Lipid: $F_{1, 439.9} = 38.61$; $p < 0.001$; Lipid^2: $F_{1, 421.9} = 3.52$; $p = 0.061$; Fig. 5.4; Appendix 4: Table S4.9), which was not consistent over time (Wald test; Batch*Lipid: $F_{8, 623.6} = 4.51$; $p < 0.001$; Batch*Lipid^2: $F_{8, 624.7} = 3.65$; $p < 0.001$; Fig. 5.4; Appendix 4: Table S4.10) with the effect of lipid becoming non-linear for some batches (see Appendix 4: Table S4.10). There was no overall effect of protein intake or an interaction between protein and lipid (all $p > 0.1$, see Appendix 4: Table S4.9), however there was evidence of a non-linear effect of protein at some time points (Batch*Protein^2: $F_{8, 641.7} = 2.00$; $p = 0.045$; Batch*Protein: $F_{8, 633.9} = 1.81$; $p = 0.072$; Fig. 4.3; Appendix 4: Table S4.10), particularly final assessment (batch 10, see Appendix 4: Table S4.10).

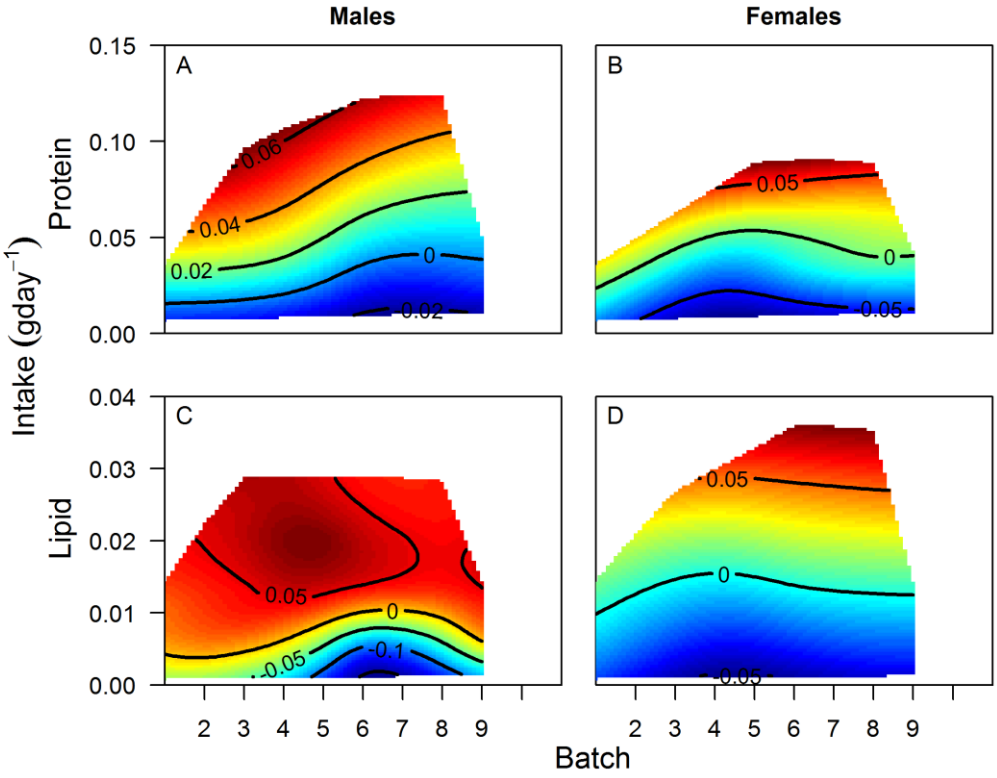

Figure 5.4 Non-parametric thin-plate spline contour visualisations for the effect of protein and lipid intake (gday^{-1}) on fish condition across batches. A positive value represents better than average condition, negative is worse than average. Panel response surfaces as follows: (A) effect of protein on male condition, (B) effect of protein on female condition, (C) effect of lipid on male condition, and (D) effect of lipid on female condition. There were significant differences in the effect of protein ($p = 0.005$) and lipid ($p = 0.033$) on the sexes.

Females

Female condition did not differ between treatments at the start of the experiment (LME; Diet: $\chi^2 = 3.36$; $p = 0.067$; Level: $\chi^2 = 0.89$; $p = 0.345$; Appendix 4: Fig.S4.3). As with males, there was a significant effect of batch on female condition (Wald test; Batch: $F_{8, 496.9} = 7.83$; $p < 0.001$; Fig 5.4; Appendix 4: Table S4.11 & Fig. S4.3). There was a significant linear effect of protein intake on female condition, with increasing protein intake improving fish condition (Wald test; Protein: $F_{1, 510.2} = 9.65$; $p = 0.002$; Fig 5.4; Appendix 4: Table S4.11). Similarly increasing lipid intake also improved female condition (Wald test; Lipid: $F_{1, 488.8} = 8.26$; $p = 0.004$; Fig 5.4; Appendix 4: Table S4.11). However, there was no evidence of any non-linear or interaction effects (all $p > 0.08$, Appendix 4: Table S4.11) or evidence that these effects changed over time (all $p > 0.2$).

Comparing the sexes

In contrast to both weight and length, there was no evidence of differences in initial condition between the sexes (LME; $\chi^2 = 1.67$; $p = 0.197$; Fig. 5.2), however there were significant differences between the sexes in final condition (Wald test; Sex: $F_{1, 1029.2} = 25.12$; $p < 0.001$; Fig. 5.2; Table 5.5) with males being in better condition than females (mean condition ± s.e.: Males = 0.016 ± 0.012; Females = -0.029 ± 0.015; Fig. 5.2). Re-enforcing this change, was a significant interaction between batch and sex (Wald test; $F_{8,1174.6} = 13.28$; $p < 0.001$; Table 5.5).

Table 5.5 Outputs from model of the sex differences in the effect macronutrient intake on condition index. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test. For sex comparisons females are the reference level.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	0.013 (0.010)	2.43	1, 1018.6	0.119
Batch 2	0 (NA)			
Batch 3	-0.017 (0.009)			
Batch 4	-0.030 (0.012)			
Batch 5	-0.058 (0.012)			
Batch 6	-0.086 (0.017)			
Batch 7	0.001 (0.021)			
Batch 8	-0.050 (0.020)			
Batch 9	-0.031 (0.020)			
Batch 10	-0.018 (0.019)	3.62	8, 1177.6	< 0.001
Protein	0.020 (0.007)	2.51	1, 979.4	0.113
Lipid	0.028 (0.012)	21.47	1, 1607.6	< 0.001
Lipid^2	-0.040 (0.013)	8.49	1, 1151.4	0.004
Sex (male)	-0.005 (0.013)	25.12	1, 1029.2	< 0.001
Batch 2*Lipid	0 (NA)			
Batch 3*Lipid	0.007 (0.009)			
Batch 4*Lipid	0.020 (0.011)			
Batch 5*Lipid	0.030 (0.012)			
Batch 6*Lipid	0.020 (0.015)			
Batch 7*Lipid	0.065 (0.016)			
Batch 8*Lipid	0.043 (0.015)			
Batch 9*Lipid	0.030 (0.015)			
Batch 10*Lipid	0.043 (0.019)	3.08	8, 1184.8	0.002
Sex (m)*Lipid	0.025 (0.009)	4.55	1, 992.9	0.033
Sex (m)*Protein	-0.021 (0.010)	7.91	1, 939.1	0.005
Batch 2*Sex (m)	0 (NA)			
Batch 3*Sex (m)	0.029 (0.012)			
Batch 4*Sex (m)	0.032 (0.016)			
Batch 5*Sex (m)	0.074 (0.017)			
Batch 6*Sex (m)	0.130 (0.024)			
Batch 7*Sex (m)	-0.085 (0.027)			
Batch 8*Sex (m)	0.023 (0.026)			
Batch 9*Sex (m)	-0.007 (0.025)			
Batch 10*Sex (m)	0.048 (0.021)	13.28	8, 1174.6	< 0.001

There were significant differences in the effect of macronutrients on the sexes with lipid improving condition more in males than in females (Wald test; Sex (m)*Lipid: $F_{1, 992.9} = 4.55$; $p = 0.033$; Fig 5.4; Table 5.5) but protein improving condition to a greater extent in females (Wald test; Sex (m)*Protein: $F_{1, 939.1} = 7.91$; $p = 0.005$; Fig 5.4; Table 5.5). However there was no evidence that these effects changed with batch (Wald test; Batch*Sex*Protein: $F_{8, 1130.9} = 0.72$; $p = 0.677$; Batch*Sex*Lipid: $F_{8, 1110.4} = 0.81$; $p = 0.693$).

5.4.3 Swimming endurance

There were no significant effects of macronutrient intake on trial 1 swimming endurance in either males or females (all $p > 0.1$; Appendix 4; Table S4.12 & Fig. S4.4). Similarly, there was no effect of weight or water temperature in either sex (all $p > 0.1$; Appendix 4; Table S4.12). However, there were significant differences in swimming endurance between the sexes (MCMCglmm: posterior mean = 1.527; 95% C.I. = 0.373 to 2.551; $p = 0.012$; Fig. 5.5; Table 5.6), with males swimming approximately twice as long as females (mean swim time (s) ± s.e.: Males = 122.57 ± 16.86; Females = 58.53.57 ± 10.05; Fig. 5.5). As there were no effects of macronutrients in either sex, we did not test for any sex specific macronutrient effects.

Swimming endurance for trial 2 mirrored those of trial 1, with no effect of macronutrient intake (all $p > 0.3$; Appendix 4; Table S4.13 & Fig. S4.4), fish weight (all $p > 0.7$; Appendix 4; Table S4.13) or water temperature (all $p > 0.4$; Appendix 4; Table S16) on swim time in either sex. However, there were still sex differences in swim time (MCMCglmm: posterior mean = 1.527; 95% C.I. = 0.373 to 2.551; $p =$

0.012; Fig. 5.5; Table 5.6), with males swimming longer than females (mean swim time (s) ± s.e.: Males = 56.49 ± 13.76; Females = 10.05 ± 5.17; Fig. 5.5).

As would be expected given the results above, there were no effects of macronutrients on the change in swim time between trial 1 and 2 in males or females (all $p > 0.1$; Appendix 4; Table S4.14). Interestingly, there was no evidence of a sex difference in the change in swim time (LME; $\chi^2 = 0.12$; $p = 0.729$; Fig. 5.5; Appendix 4; Table S4.15), suggesting that both sexes suffer the same reduction in swimming performance with age.

Table 5.6 Outputs from model of sex differences in the effect of protein and lipid intake on swimming endurance. Model outputs are from MCMCglmm models (Poisson distribution, see above). Linear estimates come from a model containing only linear terms, non-linear estimates come from a model containing all linear and non-linear terms.

	Trial 1			Trial 2		
	Posterior Mean	95% CI	*p*	Posterior Mean	95% CI	*p*
(Intercept)	-0.789	-5.683 to 3.558	0.750	-11.882	-60.328 to 39.987	0.652
Protein	-0.261	-0.881 to 0.433	0.416	-0.021	-1.773 to 1.584	0.984
Lipid	-0.266	-0.933 to 0.377	0.430	-0.160	-2.060 to 1.717	0.873
Weight	0.040	-1.081 to 1.157	0.954	-1.360	-4.562 to 2.088	0.422
Sex (male)	1.527	0.373 to 2.551	0.012	4.977	1.117 to 8.786	0.012
Water Temp	-0.100	-0.459 to 0.304	0.596	0.128	-3.026 to 3.214	0.922

Trial 1: nitt = 1,300,00; thin = 1,000; burnin= 300,000; Trial 2: nitt = 1,950,00; thin = 1,000; burnin= 300,000

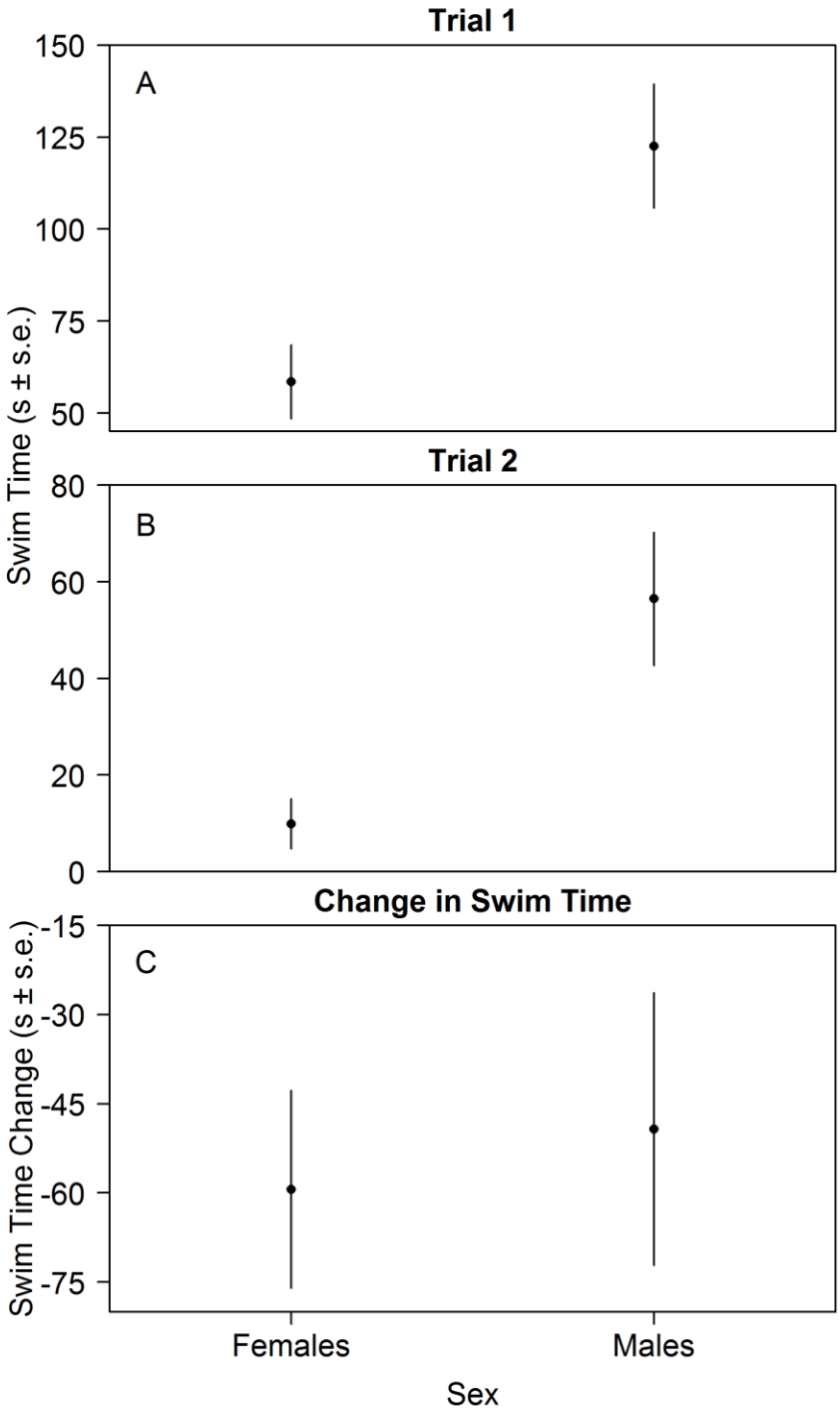

Figure 5.5 Sex differences in mean swimming endurance (s ± s.e.). Swimming time is the amount of time swimming at high current. (A) trial 1, (B) trial 2 and (C) change in swim time (difference between trials 1 and 2). There were significant differences in swimming time at both trial 1 (p = 0.012) and trial 2 (p =0.012), with males swimming longer at both trials. But the change in swim time did not differ between the sexes (p = 0.729).

5.5 Discussion

Diet is well-known to effect key fitness related traits such as growth (Partridge et al., 2005, Fontana and Partridge, 2015), with the earliest work on DR showing significant effects on body mass (Osborne et al., 1917, Mccay et al., 1935). Since the advent of the GF (reviewed Simpson and Raubenheimer, 2012), work has focussed on the key life-history traits of reproduction and lifespan, with little work exploring the effect of macronutrient intake on growth and performance. The results presented here tally with the general patterns of the few previous studies that do exist (Donaldson et al., 1956, Ruohonen et al., 2003, Ruohonen et al., 2007, Aletor et al., 2000, Sørensen et al., 2008, Huang et al., 2013, Solon-Biet et al., 2014), particularly those in mice, where a more balanced intake of protein : carbohydrate maximise growth (Sørensen et al., 2008, Huang et al., 2013, Solon-Biet et al., 2014). Here, intermediate intakes of both protein and lipid maximised growth, both in weight and length. These results add weight to our suggestion in chapter 3, that no one macronutrient can maximise growth. Rather a balanced intake of protein, lipid and energy density are required for individuals to grow at a maximal rate.

Previous work has focused on reproduction and lifespan owing to the suggested shift in the resolution of the lifespan – reproduction trade-off under DR (Shanley and Kirkwood, 2000). However, growth is also well known to trade-off with the lifespan (Charnov, 2001). Thus an interesting comparison to draw is the effect of macronutrient intakes on growth and on mortality (see Chapter 4). In Chapter 4 we showed that male mortality risk is lowest on diets with an intermediate protein : lipid, driven by a strong non-linear effect of lipid. Whereas female mortality risk was changeable, being lowest on high protein : lipid intakes in early life but

being reduced by low protein : lipid intakes in late life (see Chapter 4). Given the suggested trade-off between growth and lifespan (Charnov, 2001), it could be expected that diets which minimise growth should also maximise lifespan. This was supported by early work in calorie restriction which showed mice with the reduced growth had the longest lifespan (Osborne et al., 1917, Mccay et al., 1935), but the results presented here do not completely support this. In males, intermediate lipid intakes improved both mortality risk and growth. Furthermore, in female early life, protein intake has a positive effect on both growth and mortality risk. On the other hand, in female later life there is some evidence of a trade-off between growth and lifespan, with growth maximised by intermediate protein : lipid intakes but mortality risk lowest on low protein : lipid intakes. However, these results are difficult to compare as the effect of macronutrient intake on growth in both sexes, and female mortality risk, changes across time.

We find significant differences in condition between the sexes, with males generally being in better condition than females. Interestingly, this difference was not present prior to the initiation of diet treatments. Given the sex specific effects of macronutrient intake on condition, we suggest this is due to differential utilisation of ingested macronutrients between the sexes. As male three-spine sticklebacks have higher adiposity than females (see Chapter 3), we suggest males utilise less resources for growth, ensuring high energy reserves and better overall condition. In contrast, females invest heavily in growth, growing both longer and heavier than males, but with fewer energy stores (see Chapter 3). This difference may be due to different reproductive behaviours exhibited by the sexes. For females, there is a well-known association between size and egg production (Wootton, 1973), with larger females

producing larger clutches and thus achieving higher fitness. However, males may require greater energy reserves than females, owing to their energetically costly and time consuming reproductive behaviours (such as egg fanning) preventing males form foraging fully during the breeding season (Rohwer, 1978 and Chapter 3). Thus, a male who is in better condition with higher fat deposits needs to forage less, can invest more in reproduction, and thus achieves higher fitness.

The results presented here, coupled with those in Chapters 3 and 4, suggest a possible link between lipid intake, adiposity, health and lifespan in male sticklebacks. Intermediate lipid intakes result in higher adiposity and better overall health (as indicated by improved condition above) and males on these diets have reduced risk of mortality. This contradicts the suggestion of a relationship where a reduction in adiposity leads to an increase in lifespan (Barzilai et al., 1998, Picard and Guarente, 2005, Muzumdar et al., 2008). However, a recent study in mice found similar results, with mice on low protein, high carbohydrate diets having increased adiposity, but higher lifespan (Solon-Biet et al., 2014). Taken together these results begin to question the widespread dogma that high fat diets are bad for health and lifespan. It would be interesting to see how other measures of health are effected by high fat, or high carbohydrate, diets and in particular, whether these diets improve all measures of health. This would show if individuals on high fat diets live healthier, as well as longer lives.

A further interesting question would be, what would happen to male condition, if males were to undergo fluctuating food availability. We have suggested that the increase in adiposity and high condition seen in male sticklebacks is a result of their reproductive activity, which prevents them from foraging during the breeding

season (Rohwer, 1978). However here, males were provided with food throughout the breeding season, with possibly even the 50% treatment being a higher provisioning level than males typically experience in the wild during the breeding season. An interesting further study, would be to repeat this experiment up to the start of the breeding season, but then to severely restrict nutrient intake for all males throughout the breeding season. One possible outcome would be an exaggeration of the difference reported here, i.e. the difference in condition between males on the high lipid diets and those on the low lipid diets would increase. Alternatively, the differences reported here could remain the same, but male condition in all treatments would reduce, and the difference between males and females would return to zero.

We found no difference in the effect of macronutrient intake on swimming performance. These results have striking implications. Firstly, they could suggest that although CR is well known to effect activity and performance (e.g. Ingram et al., 1987, Means et al., 1993), these effects are not recaptured through DR via macronutrient manipulation. This could be because, despite diets being suboptimal in terms of protein : lipid ratio, they were in plentiful supply. Therefore, individuals were not under resource limitation thus there may not have been an effect on swimming endurance. However, we did not detect an effect of caloric intake on swimming performance either, which suggests that DR has no effect on swimming performance in sticklebacks. Alternatively, these results could match those of a comprehensive study in mice, which showed that various measures of activity and endurance respond to DR differently (Mitchell et al., 2016). Although Mitchell et al (2016) look at activity, rather than physical performance, it is possible that like activity, not all measures or performance and endurance respond to DR in the same

way. Therefore here, although there was no effect of macronutrients on swimming endurance, macronutrient intake may affect an alternate measure of physical performance. It would be interesting to test how other performance measures respond to varying macronutrient intake.

We detected significant difference in swimming performance between the sexes, with males swimming longer than females, in both trials. As mentioned above (and Chapter 3) males have greater lipid reserves and are in better condition than females in all treatments. Thus, males appear to have greater energy reserves than females which may enable them to swim against a high current for longer. However, in Chapter 3, we showed a positive effect of lipid intake on adiposity. If an increase in adiposity resulted in higher swimming endurance, we would have expected to see a positive effect of lipid intake on swimming endurance. Alternatively, males may be less susceptible to the high current than females owing to their smaller size, thus are able to swim for longer. However, there was no effect of weight on swimming performance to support this. Therefore, it is possible that there are fundamental differences in swimming ability between the sexes. During the breeding season, males develop nuptial colouration which increases their risk of predation (Moodie, 1982, Whoriskey and Fitzgerald, 1985, Candolin, 1998). This dimorphism in predation risk, could have resulted in higher selection for swimming ability in male sticklebacks, leading to them being able to swim for longer than females. A previous study using this method in sticklebacks did not test for sex differences in swimming ability (Alvarez and Metcalfe, 2005), it would be interesting to have future studies explore this further, to see if these sex differences in swimming performance are a general effect in sticklebacks.

This study presents an exciting advance in our understanding of the effect of macronutrient intake on growth and performance. To our knowledge, this is the first study to use the GF to test for sex specific effects of macronutrient intake on growth and performance. We report significant differences in the effect of macronutrient intake on condition index, a proxy for health in this species. More work needs to test for sex specific effects of macronutrient intake on other measures of health and condition, in a wider range of species. We did not detect any effect of macronutrient intake on performance, despite CR being well known to effect physical performance. It is possible that DR via macronutrient manipulation does not produce the same effects on activity and performance as CR. More studies are needed which apply the GF and measure traits that link to health and underlying physiological condition as this will improve our understanding of how dietary variation influences health as well as lifespan and reproduction.

Chapter 6

General Discussion

6.1 Thesis Overview

Dietary restriction (DR) is one of the most studied dietary interventions due to its ability to extend lifespan and protect against age related declines. In this thesis, DR is defined as the reduction in food intake, either through overall calorie or specific macronutrient intake. This thesis addressed the following questions: (1) How universal is the effect of the reduction in reproduction under DR? Despite being a commonly stated effect of DR, evidence usually comes from three well cited studies (Ball et al., 1947, Chippindale et al., 1993, Chapman and Partridge, 1996), with conflicting evidence often overlooked (e.g. Kaitala, 1987, Boggs and Ross, 1993, Inness and Metcalfe, 2008). (2) Is the effect of DR reproducible in a non-model vertebrate system? Recent evidence suggests macronutrient, rather than caloric intake, underpins responses to DR (reviewed Simpson et al., 2017), however this effect has been questioned, particularly in vertebrates, with the suggestion of a confounding effect of dietary dilution (Speakman et al., 2016). To date the only vertebrate species in which this has been tested are mice (*Mus musculus*; Solon-Biet et al., 2014, Mitchell et al, 2015a), where the effects of DR a known to be more effective (Nakagawa et al., 2012). (3) What is the effect of DR, particularly via macronutrient manipulation, on other fitness related traits such as growth, condition and performance? There are a plethora of studies exploring the effect of macronutrient intake on lifespan and reproduction (reviewed Simpson et al., 2017), however other fitness traits such as growth, body composition and physical performance, are often overlooked. We addressed these questions using a combination of meta-analytic techniques and experimental regimes utilising the three-spine stickleback fish (*Gasterosteus aculeatus*).

6.2 Key findings

6.2.1 Chapter 2: Meta-analytic insights

Chapter 2 utilised a systematic review and meta-analysis to explore the generality of the effect of DR on reproduction. We found that overall DR results in a reduction in reproduction, though this is moderated by several factors. DR reduces reproduction to a greater extent in model species (yeast, nematodes, fruit flies, mice and rats (see Chapter 2 and Nakagawa et al., 2012, Moatt et al., 2016), with the suggestion of a steeper slope of reproductive decline in model species with increasing restriction. This provides one potential explanation for the greater increase in lifespan seen in model species (Nakagawa et al., 2012), if model species have a greater reduction in reproduction for a given restriction level, more resources can be diverted to somatic maintenance. We also showed that the effect of DR on reproduction varies depending on the relative cost of the reproductive trait being measured, with high and medium cost traits being reduced significantly more than low cost traits under DR. We found no evidence of sex differences in the effect of DR on reproduction when accounting for all other moderators, which conflicts with previous meta-analytic findings on lifespan, showing males receive a 20% lower extension than females (Nakagawa et al., 2012).

6.2.2 Chapter 3: Body composition

In Chapter 3, we showed that macronutrient intake has significant impacts on body composition, with individuals appearing to target a balanced, internal protein : lipid ratio. Interestingly, these results suggest that individuals are able to selectively uptake or utilise ingested macronutrients, as the rank order of protein : lipid ratio

changed from the diet to carcass. Contrary to the suggestions of the protein leverage hypothesis (Simpson and Raubenheimer, 2005), we found that stickleback body composition was predicted by lipid content of the diet, not protein content, with adiposity increasing and protein deposition decreasing, with increasing lipid content of the diet. Together these results suggest that sticklebacks alter their body composition through metabolism and excretion of excess protein. We found significant sexual dimorphism in body composition, with males having greater adiposity while females had greater bone and mineral deposits. We suggest this is due to the different reproductive behaviours exhibited by the sexes, which necessitate males having greater energy reserves than females. Finally, we found no effect of nutrition on swimming endurance, activity or testes mass.

6.2.3 Chapter 4: Mortality and reproduction

Chapter 4 confirms recent findings that macronutrient, not calorie, intake predicts lifespan and reproduction under DR. Critically this is the first experiment to demonstrate this is true even without the potentially confounding effect of dietary dilution (Speakman et al., 2016). We found significant sex differences in the effect of macronutrient intake on lifespan, with mortality risk being lower on diets containing a more balanced protein : lipid ratio in males and on low protein : lipid diets in females. Interestingly, the effect of low protein : lipid intakes on female mortality was not consistent, with high protein : lipid intakes resulting in lower mortality risks in early life, but then then higher risk in adolescence. In contrast to mortality risk, we found no evidence of sex-specific effects of macronutrient intake on reproduction, with both sexes maximising reproduction on high protein : lipid intakes. These results suggest that diet may mediate the trade-off between reproduction and lifespan

in sticklebacks, mirroring recent findings in other species (e.g. Hunt et al., 2004, Lee et al., 2008, Carey et al., 2008). Finally, despite the presence of strong sex specific patterns of reproductive senescence, we found no difference in the effect of nutrition on senescence between the sexes, although increased protein significantly reduced reproductive senescence in females but not males.

6.2.4 Chapter 5: Growth and performance

Here we present data showing significant sex differences in growth, with females tending to be both longer and heavier than males throughout life. However, there were no sex differences in the effect of macronutrient intake on growth, with diets containing a balanced ratio of protein : lipid maximising growth. In contrast to this, we found significant sex differences in the effect of macronutrient intake on condition, a measure of overall fish health. Low protein : lipid intakes were more beneficial to males, while high protein : lipid intakes were better for females. Despite there being no differences in condition prior to the start of the experiment, following the start of dietary manipulations males became in better condition than females. Finally, we show significant sex differences in performance, measured as swimming endurance, with males swimming longer than females, but no effect of macronutrient intake in either sex.

6.3 Implications and future directions

6.3.1 Calories or macronutrients

The results presented here show that stickleback lifespan is more affected by macronutrients ratio than caloric intake, with lifespan maximised on diets with a low protein content. Critically, the experiments here avoid the suggested confounding effect of dietary dilution (Speakman et al., 2016). Furthermore, these results question the suggestion of a fundamental difference in the mode of action of DR between vertebrate and invertebrate species that had been proposed to explain inconsistent results in mice (Speakman et al., 2016). It is unclear why these inconsistencies arise. One possibility is that the studies reporting no effect of protein : carbohydrate ratio do not apply the geometric framework (GF) to their dietary manipulations (e.g. Mitchell et al., 2015a, Mitchell et al., 2016). By not using the GF, these studies do not perform multiple restrictions across the same ratio of protein : carbohydrate and thus, it may be harder to detect any effect of macronutrient intake. Furthermore, the experiments of Mitchell et al. (2015a) do not measure lifespan, making any comparisons to the experiments of Solon-Biet et al. (2014, 2015) difficult. It is likely that Mitchell et al. (2015a) chose not use the GF as they were attempting to avoid the effect of dietary dilution (Speakman et al., 2016). However, as shown here, it is possible to perform sophisticated dietary manipulations using the GF while using a more classical approach to introduce restrictions in calories. An interesting future study would be to repeat the GF experiments in mice (Solon-Biet et al., 2014, Solon-Biet et al., 2015), but use intermittent feeding regimes, similar to that used here, or a restricted ration to generate the restrictions, to test whether the importance of macronutrient ratio above calorie content is replicated.

A further possible explanation for the differences seen in mice, is that in the experiments of Mitchel et al. (2015a, 2016), the mice were not under significant macronutrient imbalance. Here, we provide evidence that individuals are able to moderate their internal balance of protein : fat (Chapter 3), which we suggest is due to the metabolism and excretion of excess protein. Thus it is possible that mice were able to alter their metabolism of protein and carbohydrate to ensure sufficient levels of both macronutrients (Mitchell et al., 2015a, Mitchell et al., 2016). Critically, the studies of Mitchell et al. (2015a, 2016) were performed over a relatively short period of time, whereas the work of Solon-Biet et al (2014, 2015) were over the whole life of the mouse. Therefore, it is possible that the costs suggested to be associated with this protein metabolism (see Chapter 3) may not have been apparent in the short term studies, but were more apparent in the long term studies. Furthermore, this short term restriction may have been more than sufficient for caloric restriction to cause changes in behaviour and physiology. Thus it may not be entirely surprising that Mitchell et al. (2015a, 2016) found an effect of caloric restriction and not of macronutrient intake.

Given the small number of studies to explore the effect of macronutrient intake on lifespan and reproduction in vertebrate species, definitive conclusions are difficult to draw. To date only three studies have attempted to explore the effect of macronutrient intake on vertebrate species (Solon-Biet et al., 2014, Mitchell et al., 2015a and the works presented here) and only two have attempted to reconcile the GF with vertebrate survival: the work of Solon-Biet et al. (2014) and the works presented in Chapter 4. Furthermore, two of these studies use mice, a species where the effect of DR is known to be twice as effective as in species that are not common

laboratory models (Nakagawa et al., 2012, Moatt et al., 2016 and Chapter 2), and apply calorie restriction in different manners. Thus it is clear more studies are needed on a greater variety of vertebrate species, particularly in populations that have not been maintained in the lab for many generations. Vitally, these studies should employ the GF over the long term to provide results that are directly comparable to those in insects. Additionally, more studies that compare the effect of dietary dilution versus restriction in both vertebrate and insect studies that adopt a GF approach would also be useful.

6.3.2 Effect of macronutrient intake

The overall effects of macronutrient intake presented here generally fit well with the wider field (Hunt et al., 2004, Carey et al., 2008, Lee et al., 2008, Maklakov et al., 2008, Maklakov et al., 2009, Fanson et al., 2009, Solon-Biet et al., 2014, Solon-Biet et al., 2015, Jensen et al., 2015), as we find that reproduction is maximised on high protein : lipid intakes and mortality risk is lower on low protein : lipid intakes. We find the effect on male mortality risk is driven by a positive effect of lipid intake rather than a negative effect of protein intake, in line with much of the current literature (e.g. Lee et al., 2008, Solon-Biet et al., 2014, Jensen et al., 2015). However, contrasting many recent studies (e.g. Lee et al., 2008, Jensen et al., 2015), we do not detect a negative effect of protein intake on male mortality risk. Interestingly, we present evidence for a strong link between increasing lipid intake, increasing adiposity (Chapter 3), better overall health (condition, Chapter 5) and reduced mortality risk in male sticklebacks (Chapter 4). This is in line with recent evidence in mice, where mice on low protein : carbohydrate intakes had increased adiposity and higher survival (Solon-Biet et al., 2014). These results would counter

the suggestion that the primary mechanism through which DR works to extend lifespan is through a reduction in adiposity (Picard and Guarente, 2005, Muzumdar et al., 2008). However, studies that explore the effect of macronutrient intake on both body composition and survival are rare, particularly studies that utilise the GF to do this. Clearly if we are to better understand the link between macronutrient intake, adiposity and lifespan, more studies are required.

A further difference between the results presented here and the majority of DR literature, is the changeable effect of protein intake on female mortality risk. With mortality risk lowest on high protein : lipid diets early in life, but the more typical negative association between high protein : lipid intakes and mortality risk during adulthood. The critical difference between the study here and previous studies is the period over which intake is quantified. Typically, in previous studies, intake rates were quantified over a period of stable intake once growth had ceased. As intake rates did not stabilise until relatively late on in our experiments, a more complex analysis was required, exploring time varying effects of macronutrient intake. Thus our study is actually the only study we are aware of to test for varying effects of macronutrients across ontogeny. Interestingly a study in *Drosophila melanogaster* found that larval survival was maximised on high protein : carbohydrate intakes (Rodrigues et al., 2015), in contrast to adult *D. melanogaster* where lifespan was maximised on low protein : carbohydrate intakes (Lee et al., 2008). More studies attempting to quantify whether the effect of macronutrient intake varies across ontogeny would be useful to elucidate whether this is a general pattern.

6.3.3 Sex-specific effects

A common theme throughout this thesis was the presence of sexual dimorphism in traits. However, there were relatively few instances of sex-specific effects of macronutrient intake. One of the few instances of a sex-specific effect of macronutrient intake was on mortality risk. Male mortality risk was strongly influenced by lipid intake, whereas female risk was affected by protein intake (although this affect was changeable). The sex-specific effect of macronutrient intake on mortality risk fits well with the findings of Maklakov et al. (2008), although here the effect was due to more fundamental differences between the sexes (see Chapter 4). We suggest that by exposing both males and females to high reproductive costs (Moatt et al., 2016 and Chapter 2), we have accentuated the differences in the effect of macronutrients on mortality risk (see Chapter 4 for discussion). However, conclusions are difficult to draw, as there are relatively few studies that explore the effect of macronutrient intake on lifespan in both sexes concurrently. Furthermore, studies that do apply DR to both sexes often don't expose the sexes to complete reproductive costs, particularly males (e.g. Jensen et al., 2015). More studies comparing the effect of diet on mortality are needed, especially studies exposing both males and females to a near complete range of reproductive costs.

The most striking difference between the sexes was in survival, with males having significantly greater survival than females. One potential explanation for this is that males were not exposed to a complete range of reproductive costs, despite our best attempts to expose them to a more complete range. In the wild, a significant cost is likely to be a result of egg fanning behaviour and parental care (Wootton, 1984). However, male sticklebacks are known to cannibalise eggs during this process

(Rohwer, 1978), which would have disrupted the dietary manipulations. Furthermore, as have suggested in Chapters 3 to 5, the reproductive behaviour of male sticklebacks is likely to severely reduce their ability to forage (Rohwer, 1978), which possibly explains why male sticklebacks have higher adiposity than females. However here, males were not restricted in their food availability throughout the breeding season. It is difficult to know how this would have impacted on the responses to macronutrient manipulation here (see Chapter 4 for discussion). However, it is likely that had males experienced these high costs, the difference in lifespan between the sexes would have been reduced. An interesting future study would be to repeat the present study and severely restrict male food availability during the breeding season. Furthermore, it would be interesting to see how DR affects a male's willingness to cannibalise their own eggs.

Additionally there were significant differences in size and condition between the sexes, with females being larger than males, but males being in better overall condition. As with adiposity, we suggest these differences are due to the different reproductive behaviours of sticklebacks. As discussed throughout this thesis, males are unlikely to be able to forage efficiently throughout the breeding season (Rohwer, 1978). Therefore, they may store a significant portion of their ingested lipid as fat, meaning less internal resources are immediately available for growth. However, females are able to forage during the breeding season and thus may require fewer energy reserves. Furthermore, larger females produce larger egg clutches, resulting in higher reproductive success (Wootton, 1973). It is therefore, advantageous for females to invest heavily in growth throughout life.

Finally we report significant sex differences in physical performance, with males swimming longer than females. It is possible that this sex difference in swimming ability is the result of the higher adiposity in males giving them more energy reserves. However, we found no effect of lipid intake despite evidence of a linear effect of lipid intake on adiposity. Alternatively, the sex difference in swimming endurance could be a result of the sexual dimorphism in size. Females have a much larger surface area than males, this may make them more susceptible to the effect of high currents. On the other hand there could be a fundamental difference in the swimming ability in males and female sticklebacks, potentially in response to predation risk. During the breeding season, male sticklebacks develop nuptial colouration which is thought to increase their predation risk (Moodie, 1982, Whoriskey and Fitzgerald, 1985, Candolin, 1998). It is possible therefore, that there has been selection on males to have a greater ability to swim faster and longer than females, in order to better escape predators. The experimental method used for assessing swimming endurance was based on previous work in sticklebacks, however, this did not test for the presence of sex difference in swimming endurance (Alvarez and Metcalfe, 2005). It would be interesting to perform further tests on this apparent dimorphism in swimming ability.

6.3.4 Meta-analytic insights

The results presented in Chapter 2 have striking implications for the suggestion of sex differences in the effect of DR as well as the design of future DR experiments. They highlight a potential bias in current design, which would make it easier to identify effects of DR in females rather than males. Typically females are exposed to a greater range of reproductive costs than males, making it more difficult

to detect any lifespan increases through DR in males. For example, female *D. melanogaster* can use the sperm from a single mating to fertilise up to 500 eggs (Lefevre and Jonsson, 1962), thus a single mating event and the subsequent production of eggs is likely to represent significant reproductive cost for a female. However, this one mating event will incur much smaller costs in males, as they are minimally exposed to high costs reproductive behaviours, such as courtship (Cordts and Partridge, 1996). Future studies must consider experimental design carefully, to ensure both sexes experience a full range of costly reproductive behaviours.

Despite the myriad of studies examining various aspects of DR, there is a relative paucity of studies exploring the effect of DR on reproduction. There are well in excess of 15,000 papers on DR, yet our searches and reasonably unrestrictive exclusion criteria yielded only 26 studies reporting direct effects of DR on reproduction. Furthermore, there was a significant imbalance in these studies, with only 7 of the 26 reporting results for males. None of these studies looked at lifespan or reproduction in both sexes in the same study, meaning direct comparison of the sexes was not possible. This represents a significant gap in the current literature and makes it difficult to draw definitive conclusions regarding the presence of sex differences in the effect of DR. A greater range of studies exploring the effect of DR on reproduction, particularly in males, are needed.

6.3.5 Growth and body composition

The results in Chapter 3 show that individuals have a striking ability to moderate their uptake and utilisation of ingested macronutrients, resulting in their body composition being vastly different from that of the diet. It is thought that

consignment to a specific diet prevents individuals from altering the ratio of macronutrients they ingest (Simpson and Raubenheimer, 1995, Simpson et al., 2004, Simpson and Raubenheimer, 2007). While this is true for ingestion of macronutrients, it is clearly not the case for the uptake or utilisation of ingested macronutrients. As suggested in Chapter 3, this change in body composition is likely due to the metabolism and excretion of excess protein, hinting that this could be one of the costs of ingesting high protein diets (see Fanson et al., 2012). Currently there are relatively few studies using the GF to investigate the effect of macronutrient intake on body composition, making it difficult to tell if this effect is common among other species. More studies using a greater variety of species, particularly vertebrate species, need to be carried out if the links between macronutrient intake and body composition are to be revealed.

The results presented in Chapters 3 and 5 suggest that no one dietary macronutrient can maximise growth rates, with highest growth achieved on a balanced protein : lipid intake containing high levels of both macronutrients as well as calorie content. Interestingly, these results suggest that the three key life-history traits of growth, reproduction and lifespan are maximised at different points in the nutrient landscape. With lifespan maximised at low protein : non-lipid intakes, reproduction peaking at high protein : lipid intakes and growth being highest at more balanced protein : lipid ratios containing high levels of both protein and lipid. Interestingly, fitness is thought to be maximised at these intermediate levels (e.g. see Lee et al., 2008), as a result of an optimisation of the diet mediated trade-off between survival and reproduction. Early work on calorie restriction suggested a link between reduced body size and extended lifespan (e.g. Osborne et al., 1917, Mccay et al.,

1935), however here, the diet which maximised growth did not minimise lifespan. More studies are needed that measure both growth and lifespan to better understand the trade-off between the two under macronutrient manipulation.

Given our suggestion of a changing effect of macronutrient intake on mortality over time (see above), it would be interesting to see whether the diet which maximises fitness also differs depending on stage of life. For example, in stickleback females, clutch size is strongly influenced by body size (Wootton, 1973), thus in early life, growth is likely to be important. Furthermore, in sticklebacks it is well known that predation focuses on smaller individuals (Reimchen, 1991), thus although diets which maximise growth may not minimise intrinsic mortality, they may minimise extrinsic mortality. Therefore in early life, there may be a trade-off between growth and lifespan, with diets increasing growth being favoured. However, during the breeding season when there is little to no growth, the expected trade-off between reproduction and lifespan will be seen. Furthermore, as mentioned above, the ratio of protein : carbohydrate maximising larval survival and adult lifespan are very different in *D. melanogaster* (Lee et al., 2008, Rodrigues et al., 2015). It would be interesting to see if there are more cases where different intakes are favoured at different developmental stages, particularly in organisms with determinant growth.

6.3.6 Physical performance

The results of this thesis suggest there is little to no effect of macronutrient intake on performance. These results could suggest that the well-known effect of caloric restriction on performance (e.g. Ingram et al., 1987, Means et al., 1993,

reviewed Speakman and Mitchell, 2011), is not repeatable through macronutrient manipulations. Alternatively it could confirm the suggestion that alternative measures of performance are affected by DR differently (Mitchell et al., 2016) and it was merely the measures used here that were not affected by macronutrient intake. Conclusions are impossible to draw, however, as to the best of our knowledge, these are the only studies to attempt to reconcile the GF with measures of physical performance. As the advent of the GF is relatively recent, this is perhaps unsurprising, with the majority of research focussing on key life-history traits such as reproduction and lifespan. None the less, the GF must be applied to a range of health and fitness related traits if we are to understand the wider reaching effects of DR and especially whether restriction of particular macronutrient leads to a longer *and* healthier life or just longer.

6.4 Conclusions

The works presented in this thesis represent the first use of the GF in a non-model vertebrate system and critically the first application avoiding the potentially confounding effect of dietary dilution (see Speakman et al., 2016). Furthermore, these works are one of the few studies that allows direct comparisons between the sexes in the effect of macronutrient intake. The results are in line with current research suggesting that macronutrient availability, rather than caloric content, underpins responses to DR (reviewed Simpson et al., 2017). Furthermore, these data support previous findings suggesting a link between increasing adiposity, health and lifespan (Solon-Biet et al., 2014). We find significant sex differences in the effect of macronutrient intake or mortality risk, but not on reproduction. These results highlight the lack of studies in vertebrate species that utilise the GF and a lack of

studies exploring the effect of macronutrient intake on wider fitness related measures, such as body composition and physical performance. We provide the first quantitative assessment of the generality of the effect of DR on reproduction and provide evidence for a significant model species bias. However, these results imply that previous suggestions of sex differences in responses to DR (Nakagawa et al., 2012) are the result of experimental design, rather than genuine difference between the sexes, which will be an important consideration for future DR studies. In general, these results support the suggestion of an evolutionary conserved mechanism of DR, conflicting with the more recent suggestion of species specific mode of action (Speakman et al., 2016). Thus, these results reaffirm the possibility that DR, or DR mimetics, may be useful interventions to improve health and lifespan in humans.

References

Adler, M. I. & Bonduriansky, R. 2014. Why do the well-fed appear to die young? A new evolutionary hypothesis for the effect of dietary restriction on lifespan. *BioEssays*, 36, 439-450.

Aletor, V., Hamid, I., Niess, E. & Pfeffer, E. 2000. Low-protein amino acid-supplemented diets in broiler chickens: effects on performance, carcass characteristics, whole-body composition and efficiencies of nutrient utilization. *Journal of the Science of Food and Agriculture,* 80**,** 547-554.

Alvarez, D. & Metcalfe, N. 2005. Catch-up growth and swimming performance in threespine sticklebacks (*Gasterosteus aculeatus*): seasonal changes in the cost of compensation. *Canadian Journal of Fisheries and Aquatic Sciences,* 62**,** 2169-2176.

Andrews, R. & Ørskov, E. 1970. The nutrition of the early weaned lamb: I. the influence of protein concentration and feeding level on rate of gain in body weight. *The Journal of Agricultural Science,* 75**,** 11-18.

Anson, R. M., Jones, B. & De Cabod, R. 2005. The diet restriction paradigm: a brief review of the effects of every-other-day feeding. *Age,* 27**,** 17-25.

Austad, S. N. 1989. Life extension by dietary restriction in the bowl and doily spider, *Frontinella pyramitela*. *Experimental Gerontology,* 24**,** 83-92.

Austad, S. N. & Kristan, D. M. 2003. Are mice caloric restricted in nature. *Aging Cell,* 2**,** 201-207.

Ball, Z. B., Barnes, R. H. & Visscher, M. B. 1947. The effects of dietary caloric restriction on maturity and senescence, with particular reference to fertility and longevity. *American Journal of Physiology,* 150**,** 1188-1192.

Barber, I. & Arnott, S. A. 2000. Split-clutch IVF: a technique to examine indirect fitness consequences of mate preferences in sticklebacks. *Behaviour,* 137**,** 1129-1140.

Barber, I., Nairn, D. & Huntingford, F. A. 2001. Nests as ornaments: revealing construction by male sticklebacks. *Behavioral Ecology,* 12**,** 390-396.

Barnes, A. I. & Partridge, L. 2003. Costing reproduction. *Animal Behaviour,* 66**,** 199-204.

Barzilai, N., Banerjee, S., Hawkins, M., Chen, W. & Rossetti, L. 1998. Caloric restriction reverses hepatic insulin resistance in aging rats by decreasing visceral fat. *Journal of Clinical Investigation,* 101**,** 1353.

Bates, D., Mächler, M., Bolker, B. & Walker, S. 2015. Fitting linear mixed-effects models using lme4. *2015,* 67**,** 48. arXiv:1406.5823v1

Bentley, K. T. & Schindler, D. E. 2013. Body condition correlates with instantaneous growth in stream-dwelling rainbow trout and arctic grayling. *Transactions of the American Fisheries Society,* 142**,** 747-755.

Bjedov, I. & Partridge, L. 2011. A longer and healthier life with TOR down-regulation: genetics and drugs. *Biochemical Society Transactions,* 39**,** 460.

Boggs, C. L. & Ross, C. L. 1993. The effect of adult food limitation on life history traits in *Speyeria Mormonia* (Lepidoptera: Nymphalidae). *Ecology,* 74**,** 433-441.

Bonduriansky, R., Maklakov, A., Zajitschek, F. & Brooks, R. 2008. Sexual selection, sexual conflict and the evolution of ageing and life span. *Functional Ecology,* 22**,** 443-453.

Boulton, K., Grimmer, A. J., Rosenthal, G. G., Walling, C. A. & Wilson, A. J. 2014. How stable are personalities? A multivariate view of behavioural variation over long and short timescales in the sheepshead swordtail, *Xiphophorus birchmanni. Behavioral Ecology and Sociobiology,* 68**,** 791-803.

Braithwaite, V. A. & Barber, I. 2000. Limitations to colour-based sexual preferences in three-spined sticklebacks (*Gasterosteus aculeatus*). *Behavioral Ecology and Sociobiology,* 47**,** 413-416.

Burger, J. M. S. & Promislow, D. E. L. 2004. Sex-specific effects of interventions that extend fly life span. *Science of Aging Knowledge Environment* 2004**,** pe30-.

Candolin, U. 1998. Reproduction under predation risk and the trade–off between current and future reproduction in the threespine stickleback. *Proceedings of the Royal Society B: Biological Sciences,* 265**,** 1171-1175.

Carey, J. R., Harshman, L. G., Liedo, P., Muller, H. G., Wang, J. L. & Zhang, Z. 2008. Longevity–fertility trade-offs in the tephritid fruit fly, *Anastrepha ludens*, across dietary-restriction gradients. *Aging Cell,* 7**,** 470-477.

Carrascal, L. M., Senar, J. C., Mozetich, I., Uribe, F. & Domenech, J. 1998. Interactions among environmental stress, body condition, nutritional status, and dominance in great tits. *The Auk***,** 727-738.

Chapman, T. & Partridge, L. 1996. Female fitness in *Drosophila melanogaster*: an interaction between the effect of nutrition and of encounter rate with males. *Proceedings of the Royal Society B: Biological Sciences,* 263**,** 755-759.

Chiba, T., Tamashiro, Y., Park, D., Kusudo, T., Fujie, R., Komatsu, T., Kim, S. E., Park, S., Hayashi, H. & Mori, R. 2014. A key role for neuropeptide Y in lifespan extension and cancer suppression via dietary restriction. *Scientific Reports,* 4**,** 4517.

Chippindale, A. K., Leroi, A. M., Kim, S. B. & Rose, M. R. 1993. Phenotypic plasticity and selection in *Drosophila* life-history evolution. I. nutrition and the cost of reproduction. *Journal of Evolutioanry Biology,* 6**,** 171-193.

Clutton-Brock, T. H. 1984. Reproductive effort and terminal investment in iteroparous animals. *The American Naturalist,* 123**,** 212-229.

Colman, R., Roecker, E., Ramsey, J. J. & Kemnitz, J. 1998. The effect of dietary restriction on body composition in adult male and female rhesus macaques. *Aging Clinical and Experimental Research,* 10**,** 83-92.

Colman, R. J., Beasley, T. M., Kemnitz, J. W., Johnson, S. C., Weindruch, R. & Anderson, R. M. 2014. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. *Nature Communications,* 5, 3557.

Cooper, T. M., Mockett, R. J., Sohal, B. H., Sohal, R. S. & Orr, W. C. 2004. Effect of caloric restriction on life span of the housefly, *Musca domestica*. *FASEB Journal,* 18, 1591-1593.

Cordts, R. & Partridge, L. 1996. Courtship reduces longevity of male *Drosophila melanogaster*. *Animal Behaviour,* 52**,** 269-278.

De Silva, S. S., Gunasekera, R. M. & Shim, K. 1991. Interactions of varying dietary protein and lipid levels in young red tilapia: evidence of protein sparing. *Aquaculture,* 95**,** 305-318.

Delimaris, I. 2013. Adverse effects associated with protein intake above the recommended dietary allowance for adults. *ISRN Nutrition,* 2013.

Devigili, A., Kelley, J. L., Pilastro, A. & Evans, J. P. 2013. Expression of pre- and postcopulatory traits under different dietary conditions in guppies. *Behavioural Ecology*, 24, 740-749.

Dhahbi, J. M., Mote, P. L., Fahy, G. M. & Spindler, S. R. 2005. Identification of potential caloric restriction mimetics by microarray profiling. *Physiological Genomics,* 23**,** 343-350.

Donaldson, W. E., Combs, G. F. & Romoser, G. L. 1956. Studies on energy levels in poultry rations. The effect of calorie-protein ratio of the ration on growth, nutrient utilization and body composition of chicks. *Poultry Science,* 35**,** 1100-1105.

Egger, M., Smith, G. D., Schneider, M. & Minder, C. 1997. Bias in meta-analysis detected by a simple, graphical test. *BMJ*, 315, 629-634.

Evan, G. I. & Vousden, K. H. 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature,* 411**,** 342-348.

Fanson, B. G., Weldon, C. W., Pérez-Staples, D., Simpson, S. J. & Taylor, P. W. 2009. Nutrients, not caloric restriction, extend lifespan in Queensland fruit flies (*Bactrocera tryoni*). *Aging Cell,* 8**,** 514-523.

Fanson, B. G., Fanson, K. V. & Taylor, P. W. 2012. Cost of reproduction in the Queensland fruit fly: Y-model versus lethal protein hypothesis. *Proceedings of the Royal Society of London B: Biological Sciences***,** rspb20122033.

Flatt, T. 2014. Plasticity of lifespan: a reaction norm perspective. *Proceedings of the Nutrition. Society*, 73, 532-542.

Fontana, L., Meyer, T. E., Klein, S. & Holloszy, J. O. 2004. Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. *Proceedings of the Natural Academy of Science USA,* 101**,** 6659-63.

Fontana, L., Partridge, L. & Longo, V. D. 2010. Extending healthy life span - from yeast to humans. *Science,* 328**,** 321-326.

Fontana, L. & Partridge, L. 2015. Promoting health and longevity through diet: from model organisms to humans. *Cell,* 161**,** 106-118.

Frischknecht, M. 1993. The breeding colouration of male three-spined sticklebacks (*Gasterosteus aculeatus*) as an indicator of energy investment in vigour. *Evolutionary Ecology,* 7**,** 439-450.

Genade, T., Benedetti, M., Terzibasi, E., Roncaglia, P., Valenzano, D. R., Cattaneo, A. & Cellerino, A. 2005. Annual fishes of the genus Nothobranchius as a model system for aging research. *Aging cell,* 4**,** 223-233.

Gerhard, G. S. 2003. Comparative aspects of zebrafish (*Danio rerio*) as a model for aging research. *Experimental Gerontology,* 38**,** 1333-1341.

Gerhard, G. S. 2007. Small laboratory fish as models for aging research. *Ageing Research Reviews,* 6**,** 64-72.

Gilmour, A. R., Gogel, B., Cullis, B., Thompson, R. & Butler, D. 2009. ASReml user guide release 3.0. *VSN International Ltd, Hemel Hempstead, UK*.

Gosler, A. & Harper, D. 2000. Assessing the heritability of body condition in birds: a challenge exemplified by the great tit Parus major (*Aves*). *Biological Journal of the Linnean Society,* 71**,** 103-117.

Guillaume, J. 2001. *Nutrition and feeding of fish and crustaceans*, Springer Science & Business Media.

Hadfield, J. & Nakagawa, S. 2010. General quantitative genetic methods for comparative biology: phylogenies, taxonomies and multi-trait models for continuous and categorical characters. *Journal of Evolutionary Biology*, 23, 494-508.

Hadfield, J. D. 2010. MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. *Journal of Statistical Software*, 33, 1-22.

Hambly, C. & Speakman, J. R. 2005. Contribution of different mechanisms to compensation for energy restriction in the mouse. *Obesity Research,* 13**,** 1548-1557.

Hambly, C., Mercer, J. G. & Speakman, J. R. 2007. Hunger does not diminish over time in mice under protracted caloric restriction. *Rejuvenation Research,* 10**,** 533-542.

Hansen, M., Taubert, S., Crawford, D., Libina, N., Lee, S. J. & Kenyon, C. 2007. Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*. *Aging Cell,* 6**,** 95-110.

Hariri, N. & Thibault, L. 2010. High-fat diet-induced obesity in animal models. *Nutrition Research Reviews,* 23**,** 270-299.

Harper, J. M., Leathers, C. W. & Austad, S. N. 2006. Does caloric restriction extend life in wild mice. *Aging Cell,* 5**,** 441-449.

Harrison, D. & Archer, J. 1987. Genetic differences in effects of food restriction on aging in mice. *The Journal of Nutrition,* 117**,** 376-382.

Hayflick, L. 1998. How and why we age. *Experimental Gerontology,* 33**,** 639-653.

Heaney, R. P. 1998. Excess dietary protein may not adversely affect bone. *The Journal of Nutrition,* 128**,** 1054-1057.

Helland, S. & Grisdale-Helland, B. 1998. Growth, feed utilization and body composition of juvenile Atlantic halibut (*Hippoglossus hippoglossus*) fed diets differing in the ratio between the macronutrients. *Aquaculture,* 166**,** 49-56.

Hempenstall, S., Picchio, L., Mitchell, S. E., Speakman, J. R. & Selman, C. 2010. The impact of acute caloric restriction on the metabolic phenotype in male C57BL/6 and DBA/2 mice. *Mechanisms of Ageing and Development,* 131**,** 111-118.

Higgins, J. P., Thompson, S. G., Deeks, J. J. & Altman, D. G. 2003. Measuring inconsistency in meta-analyses. *BMJ*, 327, 557-560.

Huang, X., Hancock, D. P., Gosby, A. K., Mcmahon, A. C., Solon, S., Le Couteur, D. G., Conigrave, A. D., Raubenheimer, D. & Simpson, S. J. 2013. Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice. *Obesity,* 21**,** 85-92.

Hunt, J., Brooks, R., Jennions, M. D., Smith, M. J., Bentsen, C. L. & Bussière, L. F. 2004. High-quality male field crickets invest heavily in sexual display but die young. *Nature,* 432**,** 1024-1027.

Ingram, D. K., Weindruch, R., Spangler, E. L., Freeman, J. R. & Walford, R. L. 1987. Dietary restriction benefits learning and motor performance of aged mice. *Journal of Gerontology,* 42**,** 78-81.

Ingram, D. K., Zhu, M., Mamczarz, J., Zou, S., Lane, M. A., Roth, G. S. & Decabo, R. 2006. Calorie restriction mimetics: an emerging research field. *Aging Cell,* 5**,** 97-108.

Inness, C. L. & Metcalfe, N. B. 2008. The impact of dietary restriction, intermittent feeding and compensatory growth on reproductive investment and lifespan in a short-lived fish. *Proceedings of the Royal Society B: Biological Sciences,* 275**,** 1703-8.

Iwasaki, K., Gleiser, C. A., Masoro, E. J., Mcmahan, C. A., Seo, E.-J. & Yu, B. P. 1988. Influence of the restriction of individual dietary components on longevity and age-related disease of Fischer rats: the fat component and the mineral component. *Journal of Gerontology,* 43**,** B13-B21.

Jensen, K., Mcclure, C., Priest, N. K. & Hunt, J. 2015. Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in *Drosophila melanogaster*. *Aging Cell*. 14, 605-615.

Jiang, J. C., Jaruga, E., Repnevskaya, M. V. & Jazwinski, S. M. 2000. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. *The FASEB Journal,* 14**,** 2135-2137.

Jolly, C. A. 2004. Dietary restriction and immune function. *The Journal of Nutrition,* 134**,** 1853-1856.

Kaitala, A. 1987. Dynamic life-history strategy of the waterstrider *Gerris thoracicus* as an adaptation to food and habitat variation. *Oikos,* 48**,** 125-131.

Kapahi, P., Zid, B. M., Harper, T., Koslover, D., Sapin, V. & Benzer, S. 2004. Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway. *Current Biology,* 14**,** 885-890.

Kie, J. G., White, M. & Drawe, D. L. 1983. Condition parameters of white-tailed deer in Texas. *The Journal of Wildlife Management,* 47**,** 583-594.

Kim, J. & Kaushik, S. 1992. Contribution of digestible energy from carbohydrates and estimation of protein/energy requirements for growth of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* 106**,** 161-169.

Kirkwood, T. B. L. 1977. Evolution of ageing. *Nature,* 270**,** 301-304.

Kitano, J., Mori, S. & Peichel, C. L. 2007. Sexual dimorphism in the external morphology of the threespine stickleback (*Gasterosteus aculeatus*). *Copeia,* 2007**,** 336-349.

Lakowski, B. & Hekimi, S. 1998. The genetics of caloric restriction in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences USA,* 95**,** 13091-13096.

Lande, R. & Arnold, S. J. 1983. The measurement of selection on correlated characters. *Evolution***,** 1210-1226.

Le Bourg, E. 2010. Predicting whether dietary restriction would increase longevity in species not tested so far. *Ageing Research Reviews,* 9**,** 289-97.

Lee, K. P., Simpson, S. J., Clissold, F. J., Brooks, R., Ballard, J. W., Taylor, P. W., Soran, N. & Raubenheimer, D. 2008. Lifespan and reproduction in *Drosophila*: new insights from nutritional geometry. *Proceedings of the Natural Academy of Sciences USA,* 105**,** 2498-503.

Lee, K. P. 2015. Dietary protein: carbohydrate balance is a critical modulator of lifespan and reproduction in *Drosophila melanogaster*: a test using a chemically defined diet. *Journal of Insect Physiology,* 75**,** 12-19.

Lee, W.-S., Monaghan, P. & Metcalfe, N. B. 2013. Experimental demonstration of the growth rate–lifespan trade-off. *Proceedings of the Royal Society B: Biological Sciences,* 280.

Lefevre, G. & Jonsson, U. B. 1962. Sperm transfer, storage, displacement, and utilization in *Drosophila melanogaster*. *Genetics,* 47**,** 1719-1736.

Leroi, M. A. 2001. Molecular signals versus *Loi de Balancement*. *Trends in Ecology & Evolution*, 16, 24-29.

Lessells, K. & Colegrave, N. 2001. Molecular signals or the *Loi de Balancement*? *Trends in Ecology & Evolution,* 16**,** 284-285.

Liao, C.-Y., Rikke, B. A., Johnson, T. E., Gelfond, J. a. L., Diaz, V. & Nelson, J. F. 2011. Fat maintenance is a predictor of the murine lifespan response to dietary restriction. *Aging Cell,* 10**,** 629-639.

Lipsey, M. W. & Wilson, D. B. 2001 *Practical meta-analysis*, Sage publications Thousand Oaks, CA.

Lorenzini, A., Salmon, A. B., Lerner, C., Torres, C., Ikeno, Y., Motch, S., Mccarter, R. & Sell, C. 2013. Mice producing reduced levels of insulin-like growth factor type 1 display an increase in maximum, but not mean, life span. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 69(4), 410-419.

Magwere, T., Chapman, T. & Partridge, L. 2004. Sex differences in the effect of dietary restriction on life span and mortality rates in female and male *Drosophila melanogaster*. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 59**,** B3-B9.

Mair, W., Sgro, C. M., Johnson, A. P., Chapman, T. & Partridge, L. 2004. Lifespan extension by dietary restriction in female *Drosophila melanogaster* is not caused by a reduction in vitellogenesis or ovarian activity. *Experimental Gerontology,* 39**,** 1011-1019.

Maklakov, A. A., Simpson, S. J., Zajitschek, F., Hall, M. D., Dessmann, J., Clissold, F., Raubenheimer, D., Bonduriansky, R. & Brooks, R. C. 2008. Sex-specific fitness effects of nutrient intake on reproduction and lifespan. *Current Biology,* 18**,** 1062-6.

Maklakov, A. A., Hall, M. D., Simpson, S. J., Dessmann, J., Clissold, F. J., Zajitschek, F., Lailvaux, S. P., Raubenheimer, D., Bonduriansky, R. & Brooks, R. C. 2009. Sex differences in nutrient-dependent reproductive ageing. *Aging Cell,* 8**,** 324-330.

Martin, B., Mattson, M. P. & Maudsley, S. 2006. Caloric restriction and intermittent fasting: two potential diets for successful brain aging. *Ageing Research Reviews,* 5**,** 332-353.

Masoro, E., Iwasaki, K., Gleiser, C., Mcmahan, C., Seo, E. & Yu, B. 1989. Dietary modulation of the progression of nephropathy in aging rats: an evaluation of the importance of protein. *The American Journal of Clinical Nutrition,* 49**,** 1217-1227.

Mccarter, R. J. M. 1998. Energy metabolism, nutrition and ageing. *Australasian Journal on Ageing,* 17**,** 56-59.

Mccay, C. M., Crowell, M. F. & Maynard, L. A. 1935. The effect of retarded growth upon the lenght of life span and upon the ultimate body size. *The Journal of Nutrition,* 10**,** 63-79.

Means, L. W., Higgins, J. L. & Fernandez, T. J. 1993. Mid-life onset of dietary restriction extends life and prolongs cognitive functioning. *Physiology & Behavior,* 54**,** 503-508.

Metaxakis, A. & Partridge, L. 2013. Dietary restriction extends lifespan in wild-derived populations of *Drosophila melanogaster*. *PLoS One,* 8(9), e74681

Miller, R. A., Harper, J. M., Dysko, R. C., Durkee, S. J. & Austad, S. N. 2002. Longer life spans and delayed maturation in wild-derived mice. *Experimental Biology and Medicine,* 227**,** 500-508.

Mitchell, S. E., Tang, Z., Kerbois, C., Delville, C., Konstantopedos, P., Bruel, A., Derous, D., Green, C., Aspden, R. M. & Goodyear, S. R. 2015a. The effects of graded levels of calorie restriction: I. impact of short term calorie and protein restriction on body composition in the C57BL/6 mouse. *Oncotarget*.

Mitchell, S. E., Delville, C., Konstantopedos, P., Hurst, J., Derous, D., Green, C., Chen, L., Han, J., Wang, Y. & Promislow, D. E. 2015b. The effects of graded levels of calorie restriction: II. Impact of short term calorie and protein restriction on circulating hormone levels, glucose homeostasis and oxidative stress in male C57BL/6 mice. *Oncotarget,* 6**,** 23213-23237.

Mitchell, S. E., Delville, C., Konstantopedos, P., Derous, D., Green, C. L., Chen, L., Han, J.-D. J., Wang, Y., Promislow, D. E. & Douglas, A. 2015c. The effects of graded levels of calorie restriction: III. Impact of short term calorie and protein restriction on mean daily body temperature and torpor use in the C57BL/6 mouse. *Oncotarget,* 6**,** 18314-18337.

Mitchell, S. E., Delville, C., Konstantopedos, P., Derous, D., Green, C. L., Wang, Y., Han, J.-D. J., Promislow, D. E., Douglas, A. & Chen, L. 2016. The effects of graded levels of calorie restriction: V. Impact of short term calorie and protein restriction on physical activity in the C57BL/6 mouse. *Oncotarget,* 7**,** 19147-19170.

Mitteldorf, J. 2001. Can experiments on caloric restriction be reconciled with the disposable soma theory for the evolution of senescence? *Evolution,* 55**,** 1902-1905.

Moatt, J. P., Dytham, C. & Thom, M. D. F. 2013. Exposure to sperm competition risk improves survival of virgin males. *Biology Letters,* 9, 20121188.

Moatt, J. P., Dytham, C. & Thom, M. D. F. 2014. Sperm production responds to perceived sperm competition risk in male *Drosophila melanogaster*. *Physiology & Behavior,* 131**,** 111-114.

Moatt, J. P., Nakagawa, S., Lagisz, M. & Walling, C. A. 2016. The effect of dietary restriction on reproduction: a meta-analytic perspective. *BMC Evolutionary Biology,* 16**,** 1-9.

Moodie, G. 1982. Why asymmetric mating preferences may not show the direction of evolution. *Evolution,* 36**,** 1096-1097.

Muzumdar, R., Allison, D. B., Huffman, D. M., Ma, X., Atzmon, G., Einstein, F. H., Fishman, S., Poduval, A. D., Mcvei, T., Keith, S. W. & Barzilai, N. 2008. Visceral adipose tissue modulates mammalian longevity. *Aging Cell,* 7**,** 438-440.

Nakagawa, S., Lagisz, M., Hector, K. L. & Spencer, H. G. 2012. Comparative and meta-analytic insights into life extension via dietary restriction. *Aging Cell,* 11**,** 401-409.

Nakagawa, S. & Santos, E. S. 2012. Methodological issues and advances in biological meta-analysis. *Evolutionary Ecology*, 26, 1253-1274.

Nakagawa, S. & Schielzeth, H. 2013. A general and simple method for obtaining R2 from generalized linear mixed-effects models. *Methods in Ecology and Evolution*, 4, 133-142.

Near, T. J., Eytan, R. I., Dornburg, A., Kuhn, K. L., Moore, J. A., Davis, M. P., Wainwright, P. C., Friedman, M. & Smith, W. L. 2012. Resolution of ray-finned fish phylogeny and timing of diversification. *PNAS,* 109**,** 13698-13703.

Nychka, D., Furrer, R., Paige, J. & Sain, S. 2016. Fields: Tools for spatial data. Doi:10.5065/D6W957CT.

Osborne, T. B., Mendel, L. B. & Ferry, E. L. 1917. The effect of retardation of growth upon the breeding period and duration of life of rats. *Science,* 45**,** 294-295.

Park, S., Komatsu, T., Kim, S. E., Tanaka, K., Hayashi, H., Mori, R. & Shimokawa, I. 2017. Neuropeptide Y resists excess loss of fat by lipolysis in calorie-restricted mice: a trait potential for the life-extending effect of calorie restriction. *Aging Cell,* 16**,** 339-348.

Parker, G. A., Baker, R. & Smith, V. 1972. The origin and evolution of gamete dimorphism and the male-female phenomenon. *Journal of Theoretical Biology,* 36**,** 529-553.

Partridge, L., Gems, D. & Withers, D. J. 2005. Sex and death: what is the connection? *Cell,* 120**,** 461-72.

Phelan, J. P. & Rose, M. R. 2005. Why dietary restriction substantially increases longevity in animal models but won't in humans. *Ageing Research Reviews,* 4**,** 339-50.

Picard, F. & Guarente, L. 2005. Molecular links between aging and adipose tissue. *International Journal of Obesity and Related Metabolic Disorders,* 29**,** S36-S39.

Piper, M. D., Partridge, L., Raubenheimer, D. & Simpson, S. J. 2011. Dietary restriction and aging: a unifying perspective. *Cell Metabolism,* 14**,** 154-60.

Ponton, F., Wilson, K., Holmes, A., Raubenheimer, D., Robinson, K. L. & Simpson, S. J. 2015. Macronutrients mediate the functional relationship between Drosophila and Wolbachia. *Proceedings of the Royal Society of London B: Biological Sciences,* 282**,** 20142029.

R Core Team. 2017. R: A language and environment for statistical Computing. Vienna, Austria. https://R-project.org/.

Reimchen, T. E. 1991. Trout foraging failures and the evolution of body size in stickleback. *Copeia,* 1991**,** 1098-1104.

Rodrigues, M. A., Martins, N. E., Balancé, L. F., Broom, L. N., Dias, A. J., Fernandes, A. S. D., Rodrigues, F., Sucena, É. & Mirth, C. K. 2015. *Drosophila melanogaster* larvae make nutritional choices that minimize developmental time. *Journal of Insect Physiology,* 81**,** 69-80.

Rohwer, S. 1978. Parent cannibalism of offspring and egg raiding as a courtship strategy. *The American Naturalist,* 112**,** 429-440.

Ross, M. H. Protein, calories and life expectancy. *Federation Proceedings*, 1959, 1190-1207.

Ross, M. H. 1961. Length of life and nutrition in the rat. *Journal of Nutrition,* 75**,** 197-210.

Ross, M. H. & Bras, G. 1971. Lasting influence of early caloric restriction on prevalence of neoplasms in the rat. *Journal of the National Cancer Institute,* 47**,** 1095-1114.

Rubinsztein, D. C., Mariño, G. & Kroemer, G. 2011. Autophagy and aging. *Cell,* 146**,** 682-695.

Rubio, V. C., Sánchez-Vázquez, F. J. & Madrid, J. A. 2003. Macronutrient selection through postingestive signals in sea bass fed on gelatine capsules. *Physiology & Behavior,* 78**,** 795-803.

Ruohonen, K., Koskela, J., Vielma, J. & Kettunen, J. 2003. Optimal diet composition for European whitefish (*Coregonus lavaretus*): analysis of growth and nutrient utilisation in mixture model trials. *Aquaculture,* 225**,** 27-39.

Ruohonen, K., Simpson, S. J. & Raubenheimer, D. 2007. A new approach to diet optimisation: a re-analysis using European whitefish (*Coregonus lavaretus*). *Aquaculture,* 267**,** 147-156.

Russell, J., Epling, W., Pierce, D., Amy, R. & Boer, D. 1987. Induction of voluntary prolonged running by rats. *Journal of Applied Physiology,* 63**,** 2549-2553.

Sanchez-Vazquez, F. J., Yamamoto, T., Akiyama, T., Madrid, J. A. & Tabata, M. 1999. Macronutrient self-selection through demand-feeders in rainbow trout. *Physiology & Behaviour,* 66**,** 45-51.

Selman, C., Phillips, T., Staib, J. L., Duncan, J. S., Leeuwenburgh, C. & Speakman, J. R. 2005. Energy expenditure of calorically restricted rats is higher than predicted from their altered body composition. *Mechanisms of Ageing and Development,* 126**,** 783-793.

Selman, C. 2014. Dietary restriction and the pursuit of effective mimetics. *Proceedings of the Nutrition Society,* 73**,** 260-270.

Shanley, D. P. & Kirkwood, T. B. L. 2000. Calorie restriction and aging: a life-history analysis. *Evolution,* 54**,** 740-750.

Shanley, D. P., Aw, D., Manley, N. R. & Palmer, D. B. 2009. An evolutionary perspective on the mechanisms of immunosenescence. *Trends in Immunology,* 30**,** 374-381.

Simons, M. J. P., Koch, W. & Verhulst, S. 2013. Dietary restriction of rodents decreases aging rate without affecting initial mortality rate – a meta-analysis. *Aging Cell,* 12**,** 410-414.

Simpson, S. J. & Raubenheimer, D. 1995. The geometric analysis of feeding and nutrition: a user's guide. *Journal of Insect Physiology,* 41**,** 545-553.

Simpson, S. J., Sibly, R. M., Lee, K. P., Behmer, S. T. & Raubenheimer, D. 2004. Optimal foraging when regulating intake of multiple nutrients. *Animal Behaviour,* 68**,** 1299-1311.

Simpson, S. & Raubenheimer, D. 2005. Obesity: the protein leverage hypothesis. O*besity Reviews,* 6**,** 133-142.

Simpson, S. J. & Raubenheimer, D. 2007. Caloric restriction and aging revisited: the need for a geometric analysis of the nutritional bases of aging. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 62**,** 707-713.

Simpson, S. J. & Raubenheimer, D. 2009. Macronutrient balance and lifespan. *Aging (Albany NY),* 1**,** 875.

Simpson, S. J. & Raubenheimer, D. 2012. *The nature of nutrition: a unifying framework from animal adaptation to human obesity*. Princeton University Press.

Simpson, S. J., Le Couteur, D. G., Raubenheimer, D., Solon-Biet, S. M., Cooney, G. J., Cogger, V. C. & Fontana, L. 2017. Dietary protein, aging and nutritional geometry. *Ageing Research Reviews*. doi.org/10.1016/j.arr.2017.03.001

Skorupa, D. A., Dervisefendic, A., Zwiener, J. & Pletcher, S. D. 2008. Dietary composition specifies consumption, obesity, and lifespan in *Drosophila melanogaster*. *Aging Cell,* 7**,** 478-490.

Solon-Biet, S. M., Mcmahon, A. C., Ballard, J. W. O., Ruohonen, K., Wu, L. E., Cogger, V. C., Warren, A., Huang, X., Pichaud, N. & Melvin, R. G. 2014. The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in *ad libitum*-fed mice. *Cell Metabolism,* 19**,** 418-430.

Solon-Biet, S. M., Walters, K. A., Simanainen, U. K., Mcmahon, A. C., Ruohonen, K., Ballard, J. W. O., Raubenheimer, D., Handelsman, D. J., Le Couteur, D. G. & Simpson, S. J. 2015. Macronutrient balance, reproductive function, and lifespan in aging mice. *Proceedings of the National Academy of Sciences USA,* 112**,** 3481-3486.

Sørensen, A., Mayntz, D., Raubenheimer, D. & Simpson, S. J. 2008. Protein-leverage in mice: the geometry of macronutrient balancing and consequences for fat deposition. *Obesity,* 16**,** 566-571.

Speakman, J. R. & Hambly, C. 2007. Starving for life: What animal studies can and cannot tell us about the use of caloric restriction to prolong human lifespan. *The Journal of Nutrition,* 137**,** 1078-1086.

Speakman, J. R. & Mitchell, S. E. 2011. Caloric restriction. *Molecular Aspects of Medicine,* 32**,** 159-221.

Speakman, J. R., Mitchell, S. E. & Mazidi, M. 2016. Calories or protein? The effect of dietary restriction on lifespan in rodents is explained by calories alone. *Experimental Gerontology*, 86, 28-38.

Tarnopolsky, M. A., Atkinson, S. A., Macdougall, J. D., Chesley, A., Phillips, S. & Schwarcz, H. P. 1992. Evaluation of protein requirements for trained strength athletes. *Journal of Applied Physiology (1985),* 73**,** 1986-95.

Tatar, M. 2011. The plate half-full: status of research on the mechanisms of dietary restriction in *Drosophila melanogaster*. *Experimental Gerontology*, 46, 363-368.

Terzibasi, E., Lefrançois, C., Domenici, P., Hartmann, N., Graf, M. & Cellerino, A. 2009. Effects of dietary restriction on mortality and age-related phenotypes in the short-lived fish *Nothobranchius furzeri*. *Aging Cell,* 8**,** 88-99.

Therneau, T. M. & Grambsch, P. M. 1991. *Modeling survival data: extending the Cox model.* Springer Publishing

Thomson, C. E., Bayer, F., Crouch, N., Farrell, S., Heap, E., Mittell, E., Zurita-Cassinello, M. & Hadfield, J. D. 2017. Selection on parental performance opposes selection for larger body mass in a wild population of blue tits. *Evolution,* 71**,** 716-732.

Tinbergen, N. 1951. *The study of instinct.* New York, Clarendon Press/Oxford University Press.

Trautwein, M.D., Wiegmann, B.M., Beutel, R., Kjer, K.M. & Yeates, D.K. 2012 Advances in insect phylogeny at the dawn of the postgenomic era. *Annual Review of Entomology*, 57, 449-468.

Vergara, J. M., Robainà, L., Izquierdo, M. & De La Higuera, M. 1996. Protein sparing effect of lipids in diets for fingerlings of gilthead sea bream. *Fisheries Science,* 62**,** 624-628.

Viechtbauer, W. 2010. Conducting meta-analyses in R with the metafor package. *Journal of Statistical Software*, 36, 1-48.

Vinogradov, A. E. 1998. Male reproductive strategy and decreased longevity. *Acta Biotheoretica,* 46**,** 157-160.

Van De Pol, M. & Verhulst, S. 2006. Age-dependent traits: a new statistical model to separate within-and between-individual effects. *The American Naturalist,* 167**,** 766-773.

Walford, R. L., Harris, S. B. & Gunion, M. W. 1992. The calorically restricted low-fat nutrient-dense diet in Biosphere 2 significantly lowers blood glucose, total leukocyte count, cholesterol, and blood pressure in humans. *Proceedings of the National Academy of Sciences USA,* 89**,** 11533-11537.

Walford, R. L., Mock, D., Maccallum, T. & Laseter, J. L. 1999. Physiologic changes in humans subjected to severe, selective calorie restriction for two years in biosphere 2: health, aging, and toxicological perspectives. *Toxicological Sciences,* 52**,** 61-65.

Weed, J., Lane, M., Roth, G., Speer, D. & Ingram, D. 1997. Activity measures in rhesus monkeys on long-term calorie restriction. *Physiology & Behavior,* 62**,** 97-103.

Whoriskey, F. & Fitzgerald, G. 1985. The effects of bird predation on an estuarine stickleback (Pisces: *Gasterosteidae*) community. *Canadian Journal of Zoology,* 63**,** 301-307.

Williams, G. C. 1957. Pleiotropy, natural selection, and the evolution of senescence. *Evolution,* 11**,** 398-411.

Williams, G. C. 1966. Natural selection, the costs of reproduction, and a refinement of Lack's Principle. *The American Naturalist*, 100, 916, 687-690.

Wootton, R. J. 1973. Fecundity of the three-spined stickleback, *Gasterosteus aculeatus*. *Journal of Fish Biology,* 5**,** 683-688.

Wootton, R. J. 1984. *A functional biology of sticklebacks.* University of California Press.

Wullschleger, S., Loewith, R. & Hall, M. N. 2006. TOR signaling in growth and metabolism. *Cell,* 124**,** 471-484.

Yu, B. P., Masoro, E. J. & Mcmahan, C. A. 1985. Nutritional influences on aging of Fischer 344 rats: I. Physical, metabolic, and longevity characteristics. *Journal of Gerontology,* 40**,** 657-670.

Appendices

Appendix 1:

The effect of dietary restriction on reproduction: a meta-analytic perspective.

As published:

Moatt, J.P., Nakagawa, S., Lagisz, M. & Walling, C. A. 2016. The effect of dietary restriction on reproduction: a meta-analytic perspective. *BMC Evolutionary Biology*. **16**:199.

S1.1. Supplementary Methods

Collecting studies on dietary restriction (DR) and reproduction

The data for the meta-analysis were collected through a search of ISI Web of Science and Scopus during December 2013 by J. P. Moatt using the search string 'diet*/calor* + restriction + reproduction/ fertility/fecundity'. Backward and forward searching was carried out to identify additional papers that were missed in the main database search, as well the authors' own literature collections on the subject were considered. Authors of interest were contacted in attempt to obtain unpublished data for inclusion in the analysis. However, no unpublished data matching the selection criteria were found. Grey literature and non-English language papers were also considered during selection. Of the 1,679 unique papers the search returned, papers were selected which had applied DR and reported some measure of reproduction, for treated (DR) and control females or males (usually presented as a means and standard errors). Papers were included if they met the following criteria:

1. Papers must be original empirical data using real animals, not reviews or computer simulations.
2. Animals must not be mutant or transgenic.

3. Degree of dietary restriction must be explicitly stated.
4. Intermittent feeding is allowed, as long as fasting period does not exceed the equivalent of every other day feeding. Feeding days must not allow compensatory gorging.
5. Information on the control groups intake must be given, and be either *ad libitum* or 100%.
6. Restriction must have been initiated prior to copulation and must remain constant throughout the course of the experiment.
7. There were no other confounding cofactors, such as resveratrol or pathogen treatment.

Additionally, we excluded studies where only measures of reproductive hormone levels were reported or information necessary for calculating effect sizes was missing (e.g. sample sizes, variances). Screening was carried out by J. P. Moatt between January and June 2014. Although the screening was carried out alone, discussion over the inclusion of a number of papers took place between C. A. Walling and J. P. Moatt.

Extracting effect size

In the majority of papers, reproductive data was presented in the main text as mean and standard error as well as sample sizes. In studies where this was not the case, authors were contacted in an attempt to obtain the relevant data. Effects sizes were then calculated using an effect size calculator (Lipsey & Wilson, 2001). Effect sizes are the standardised mean difference (SMD) Cohen's *d*, a measure of the difference in reproduction between the control and restricted

groups, standardised by the pooled standard deviation estimates from the two groups.

$$d = \frac{\overline{x}_1 - \overline{x}_2}{s}$$

X_1 = mean for control group

X_2 = mean for treatment group

s = pooled standard deviation. Calculated as below:

$$s = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$

n_1 = sample size of control group

n_2 = sample size of treatment group

s_1 = standard deviation of control group

s_2 = standard deviation of treatment group

Extracting Moderators (DR associated variables)

Methods sections from each paper were examined and any relevant moderators were extracted and recorded as follows:

- Model Species: 1 = yes, 0 = no, model species counted as the same five model species as in Nakagawa *et. al.* (2012): yeast (*Sacchromyces cerevisiae*), nematode (*Caenorhabditis elegans*), fruit fly (*Drosophila melanodaster*), mouse (*Mus musculus*) and rat (*Rattus norvegicus*).

- Strain name/type: unique strain names for a particular species (note that unique names are given for WT or the same strain names for different species).
- Sex: sex of the group *d* was extracted for (M = male, F = female).
- Food schedule: feeding regime used (D = daily, W = Weekly).
- Type of restriction being used: CNM = Calorie and nutrient manipulation, these were papers that included a number of diets of varied composition. However, these studies were only included if each diet was provided at multiple restriction levels, including a control level; FC = food concentration, where lower concentrations of the same food medium were used in treatment relative to control group; FS = feeding schedule, where restriction was implemented through a feeding schedule, as less frequent feeding than in the control group, e.g. every other day feeding vs. every day feeding; FW = food weight, where the same food was given in smaller quantities in treatment relative to control group.
- Feeding regime of control: 0 = 100% feeding, where individuals were given a set quantity and this was counted as fully fed; 1 = *ad libitum* where unrestricted access to food was allowed.
- Units of control and treatment group nutrition levels (when given): e.g., J/day/individual.
- Calories in control diet (when information provided): caloric density of the food.
- Costliness of the reproductive trait: A categorical measure that describes the degree to which the reproductive trait measured reflects the total cost

of reproduction in the species used: 1 = low cost – trait represents a relatively small fraction of the total cost of reproduction in that species, 2 = moderate cost, trait represents a moderate fraction of the total cost of reproduction in that species, 3 = high cost, trait represents the majority of the cost of reproduction in that species. This measure accounted for differences between species and sexes within species. For example, in *D. melanogaster*, ejaculate production is classed as low cost, courtship for a single mating event represents a medium cost and lifetime courtship investment is high cost, as courtship is thought to be one of the most costly aspects of reproduction for male *D. melanogaster* (Cordts & Partridge, 1996). For females, daily egg production represents a medium cost, whereas lifetime egg production is high cost, see Table S1.

- Reproductive measure examined: e.g., lifetime egg production, number of sperm.
- Units of the reproductive trait measured (where necessary): e.g., mass of eggs produced in g.
- The value of the reproductive trait being measured for the control group.
- Standard deviation of the mean for control group.
- Number of control individuals.
- Caloric value of restricted diet (when given).
- Restriction level, represented as a percentage decrease from control group: e.g. 40% restriction means treatment group give 60% of control diet.

- The value of the reproductive trait being measured for the restricted group.
- Standard deviation of the mean for restricted group.
- Number of restricted individuals.

Any other information considered relevant or important was noted. For complete records see Data S1 and for the detailed description of all the columns in the data table see Dialog S2.

Constructing phylogenetic tree

A topological (without branch lengths) phylogenetic tree was constructed for the subset of species included in this study using the Interactive Tree of Life (http://itol.embl.de/index.shtml). Polytomies among insect orders were resolved using information obtained from Trautwein *et al.* (2012).

General meta-analytic techniques

For the main analyses we used mixed effects meta-analysis (MM) or phylogenetic mixed effects meta-analysis (PMM) implemented in the *metaphor* package (Viechtbauer, 2012), version 1.9-3, and *MCMCglmm* package (Hadfield, 2010) for R (R core team (2014)). As model results we present mean standardized difference between control and restricted groups, standard errors, and 95% credible intervals (CIs). When comparing phylogenetic models to non-phylogenetic models we present the Akaike information criterion AIC, which is a model selection index, with the better model having the smaller AIC. The *R* scripts for all analyses are available as supplementary materials with this article.

Main meta-analytic models (Model 1 and 2)

Models 1 and 2 (Table S1.2) were simple models only fitting the effect size as a response variable, with the intercept as the fixed factor and the following random factors; study ID, animal (species ID), group ID (identifies cases where multiple types of reproduction traits were reported for the same groups of individuals) and effect size ID. These were to account for the main sources of non-independence between our measures. Model 1 only differed from Model 2 in that it accounted for phylogenetic variance.

Heterogeneity

A meta-analysis will inevitably bring together studies that differ in design and set up, particularly in reference to treatments, exposures and outcomes explored, this is referred to as heterogeneity (Higgins & Thompson, 2002). We must account for heterogeneity to explain the differences observed between the studies included in a meta-analysis. Here, we used an extended version of I^2 (Higgins & Thompson, 2002) as our heterogeneity statistic, which is described in Nakagawa and Santos (2012). This multi-level model extension of I^2 enables us to obtain heterogeneity due to each level or random factor.

Meta-analytic models with moderators (Models 3-11)

Our main question was to see whether investment in reproduction was decreased under DR. However, we also explored variables we thought may be important predictors of variation in the effect of DR on reproduction, known as moderators. We added each moderator separately to the main meta-analytical model (Model 2) to assess the effect of individual moderators (Models 3-7). These moderators included:

(a) whether the control group was fed a specific pre-defined amount or concentration of food (100%) or were allowed *ad libitum* access to food (only included in full models 8 - 11), (b) whether the species was one of the five model species or not (Table S1.4, Model 3), (c) which sex was being studied (Table S1.5,Model 4), (d) the linear and quadratic effect of degree of restriction (Table S1.6, Model 5), (e) the relative cost of the reproductive trait being studied (low, moderate and high, Table S1.1 for trait classification, Table S1.7 for model output, Model 6). We also fitted the interaction between model/non-model species and degree of restriction (Table S1.8, Model 7). We then created a number of full models where all moderators were fitted at the same time (Tables S1.9-S1.13,Models 8 - 11). Models 8 and 9 included all moderators and the interaction between model/non-model species and degree of restriction. Models 10 and 11 included all moderators but excluded the interaction between model/non-model species and degree of restriction. Models 9 and 11 are models which account for the phylogenetic variance.

Publication Bias

Publication bias is the favouring of statistically significant results during publication, regardless of the underlying effect size. We used two typical ways of assessing publication bias: (1) visual inspection via a funnel plot and (2) Eggers regression, which assess bias through a regression method (Egger *et al*, 1997). However, these methods assume that effect sizes are independent of each other. We therefore used meta-analytic residuals (sampling error and residuals) from our full model for Egger regression to fulfil this assumption. (Nakagawa & Santos, 2012).

S1.2 Supplementary Tables

Table S1.1 List of reproductive traits and the cost category they were assigned.

Low Cost (n=40)	**Medium Cost** (n=87)	**High Cost** (n=78)
Number of eggs fertilised (measured when only males under DR)	Testes weight, lifetime investment in sperm production	Number of females pregnant at least once in lifetime, lifetime investment in reproduction
Proportion of fertile eggs that hatch (measured when only males under DR)	Daily fecundity, high cost but not lifetime investment	Total fecundity, lifetime investment in egg production.
Pair formation when both sexes under DR, measured as proportion of birds that successfully pair	Size of 1st egg clutch, similar to above, high cost but not lifetime investment.	Reproductive effort, lifetime measure
All sperm / ejaculate composition, e.g. sperm length, ejaculate volume, proportion of live sperm etc	Date of 1st egg production, age of sexual maturity	Lifetime clutch production
Time per clutch, time to lay eggs	Gestation length, assuming more significant cost to female than litter growth/weight	Number of females reproducing during breeding season.
Mating-oviposition interval, not measuring number of eggs produced or matured in this time	Male courtship of females, known to be costly but only one reproductive behaviour measured	Sexual activity, measuring full range of male precopulatory behaviour
Foetal growth (g per day)	Egg load, females were unmated, killed and dissected.	
	Eggs counted midway through life	
Litter body mass at birth	Reproductive success for single breeding season, not lifetime reproductive success	
Egg mass, investment in single egg	Litter size, combination of egg number and provisioning of foetus	
	Number of clutches/eggs for part of life, not lifetime investment in eggs	
	Reproductive period (days), measure of single reproductive season	
	Oviposition days for single breeding season	
	Reproductive success, single breeding season	

Table S1.2 Comparing phylogenetic mixed effect model (PMM, Model 1) and non-phylogenetic mixed effect model (MM, Model 2) estimates of the effect of DR on reproduction. AIC taken from ML models.

	Effect size	SE	Lower CI	Upper CI	AIC
PMM	-0.841	0.272	-1.374	-0.308	577.33
MM	-0.841	0.272	-1.374	-0.308	579.86

Table S1.3 Table of heterogeneity statistics (I^2 values) for Models 1 and 2.

	Model 1	Model 2
Total Heterogeneity	98.65	98.65
Variance due to Phylogeny	0.0000667	NA
Variance due to Study	74.83	74.83
Variance due to Group	3.91	3.91
Residuals against sampling error	19.91	19.91

Table S1.4 Estimated effect sizes from the non-phylogenetic mixed effect model with the linear and quadratic effect of restriction as moderators (Model 5)

	Effect size	SE	Lower CI	Upper CI
Restriction	-0.016	0.003	-0.022	-0.010
Restriction^2	0.884	0.923	-0.925	2.694

Table S1.5 Estimated effect sizes from the non-phylogenetic mixed effect model with model/non-model species fitted as a moderator (Model 3).

	Effect size	SE	Lower CI	Upper CI
Model	-2.416	0.506	-3.406	-1.425
Non-model	-0.447	0.245	-0.926	0.033
Contrast	-1.969	0.562	-3.070	-0.868

Table S1.6 Estimated effect sizes from the non-phylogenetic mixed effect model with the interaction between model species and restriction fitted as moderators (Model 7)

	Effect size	SE	Lower CI	Upper CI
Restriction	-0.013	0.003	-0.020	-0.007
Model	0.769	1.035	-1.261	2.798
Restricition:Model	-0.042	0.015	-0.071	-0.012

Table S1.7 Estimated effect sizes from the non-phylogenetic mixed effect model with sex as a moderator (Model 4)

	Effect size	SE	Lower CI	Upper CI
Female	-1.051	0.316	-1.671	-0.431
Male	-0.274	0.519	-1.291	0.742
Contrast	0.776	0.608	-0.414	1.967

Table S1.7 Estimated effect sizes from the non-phylogenetic mixed effect model with cost of trait fitted as a moderator (Model 6)

	Effect size	SE	Lower CI	Upper CI
Low Cost	-0.244	0.315	-0.861	0.374
Moderate Cost	-1.050	0.288	-1.615	-0.484
High Cost	-1.124	0.298	-1.708	-0.539

Table S1.9 Estimated effect sizes from the non-phylogenetic mixed effect model with all moderators fitted, including the interaction between restriction and model species (Model 8). AIC taken from ML models.

	Effect size	SE	Lower CI	Upper CI
Year	0.034	0.018	-0.001	0.067
Ad Lib feeding	-0.173	0.434	-1.024	0.678
Restriction	-0.357	0.083	-0.520	-0.194
Model species	-1.074	0.625	-2.298	0.150
Male	-0.151	0.501	-1.132	0.830
Scaled cost	-0.252	0.094	-0.436	-0.067
Restricition:Model	-1.317	0.435	-2.169	-0.465

AIC = 528.08

Table S1.10 Estimated effect sizes from the non-phylogenetic mixed effect model with all moderators fitted, omitting the interaction between restriction and model species (Model 10). AIC taken from ML models.

	Effect size	SE	Lower CI	Upper CI
Year	0.014	0.019	-0.024	0.051
Ad Lib feeding	0.295	0.470	-0.627	1.217
Restriction	-0.390	0.084	-0.554	-0.226
Model species	-1.634	0.685	-2.977	-0.291
Male	-0.148	0.569	-1.264	-0.069
Scaled cost	-0.257	0.096	-0.446	-0.054

AIC = 537.22

Table S1.11 Estimated effect sizes from the phylogenetic mixed effect model with all moderators fitted, including the interaction between restriction and model species (Model 9). AIC taken from ML models.

	Effect size	SE	Lower CI	Upper CI
Year	0.034	0.018	-0.001	0.070
Ad Lib feeding	-0.173	0.434	-1.024	0.679
Restriction	-0.357	0.083	-0.520	-0.194
Model species	-1.074	0.625	-2.298	0.150
Male	-0.151	0.501	-1.133	0.830
Scaled cost	-0.252	0.094	-0.436	-0.067
Restricition:Model	-1.317	0.435	-2.169	-0.465

AIC = 530.08

Table S1.12 Estimated effect sizes from the phylogenetic mixed effect model with all moderators fitted, omitting the interaction between restriction and model species (Model 11). AIC taken from ML models.

	Effect size	SE	Lower CI	Upper CI
Year	0.014	0.019	-0.024	0.051
Ad Lib feeding	0.295	0.470	-0.627	1.217
Restriction	-0.390	0.084	-0.554	-0.226
Model species	-1.634	0.685	-2.977	-0.291
Male	-0.148	0.569	-1.264	0.968
Scaled cost	-0.257	0.096	-0.446	-0.069

AIC = 539.22

Table S1.13 Table of heterogeneity statistics (I^2 values) for Models 8 and 9.

	Model 8	Model 9
Total Heterogeneity	97.54	97.58
Variance due to Phylogeny	NA	0.00002
Variance due to Study	59.54	59.54
Variance due to Group	0.00006	0.00
Residuals against sampling error	38.04	38.03

S1.3 Supplementary figures

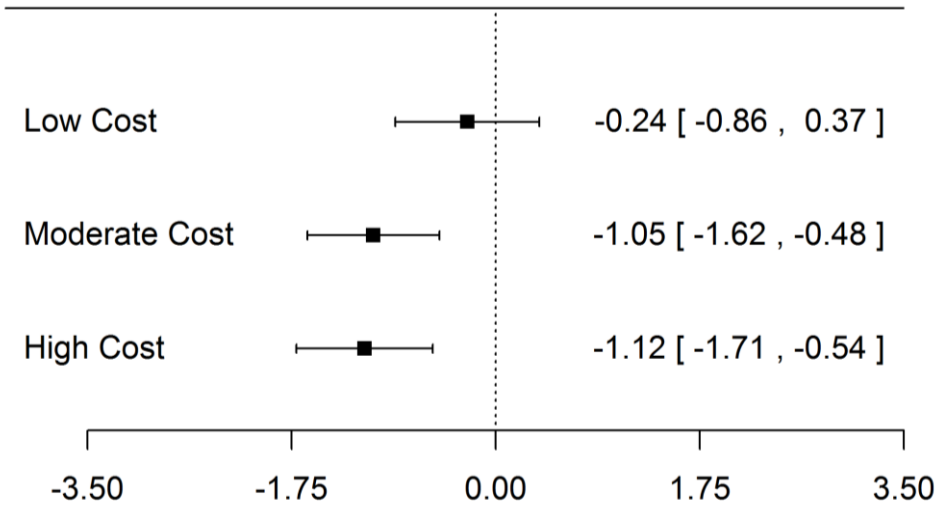

Figure S1.1 Forest plot showing effect sizes (Cohen's *d*) for the effect of dietary restriction (DR) on reproduction, for different levels of cost of reproductive trait included as a moderator. Each point represents the Cohen's *d* value for that moderator with the 95% credible intervals (CIs). High and moderate cost traits undergo a significant reduction under DR, however low cost traits do not.

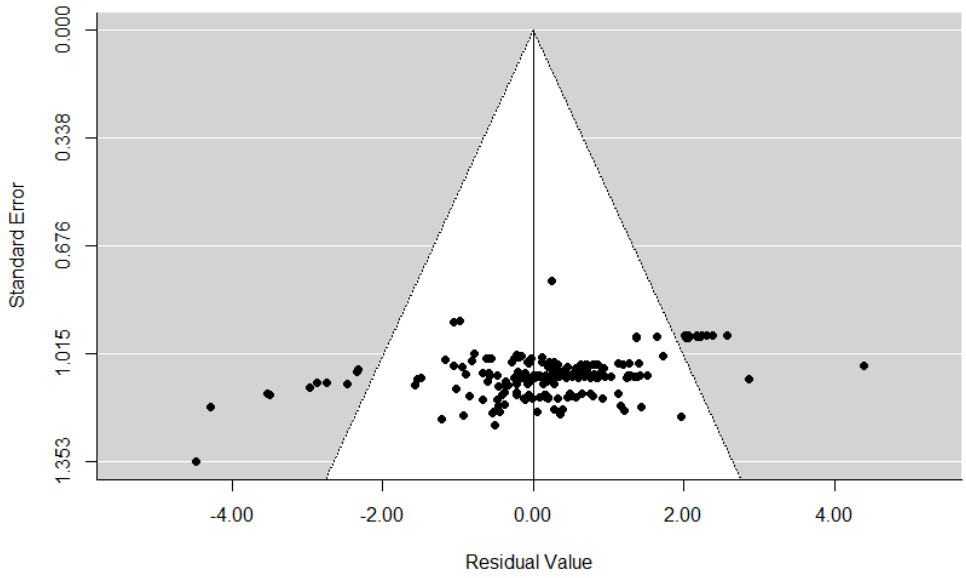

Figure S1.2 Funnel plot to allow visualisation of potential publication bias in our data set. The X axis represents the residual values from the non-phylogenetic mixed effects model containing all moderators and the interaction of restriction and model species, the Y axis represents the standard error. Publication bias indicated if data points clustered towards zero residual values as standard error decreases. Visual inspection suggests this is not the case.

Appendix 2:

Body macronutrient composition is predicted by lipid and not protein content of the diet.

S2.1 Supplementary Tables

Table S2.1 Recipe for the five diets used in this experiment. Herring meal is both a source of protein and lipid, therefore fish oil was only required in diets with high lipid contents.

Ingredient (%)	10.2 : 1	8.5 : 1	4.6 : 1	2.5 : 1	1.6 : 1
Herring Meal	90.24	41.65	79.83	69.42	41.65
Corn Starch (Filler)	6.26	54.85	11.17	13.61	38.77
Lecithin	1.00	1.00	1.00	1.00	1.00
Vitamin /mineral premix	1.00	1.00	1.00	1.00	1.00
ASTX (10% carophyll pink)	1.00	1.00	1.00	1.00	1.00
CMC binder	0.50	0.50	0.50	0.50	0.50
Fish Oil	0.00	0.00	5.50	13.47	16.08

Table S2.2 Output from post hoc Tukey analysis of model exploring the effect of diet on the final weight of fish.

Comparison	Estimate. (s.e.)	z	*p*
4.6 : 1 – 10.2 : 1	-0.061 (0.111)	-0.543	0.983
2.5 : 1 – 10.2 : 1	0.317 (0.113)	2.802	0.040
8.5 : 1 – 10.2 : 1	-0.140 (0.104)	-1.353	0.657
1.6 : 1 – 10.2 : 1	-0.005 (0.106)	-0.049	> 0.999
2.5 : 1 – 4.6 : 1	0.377 (0.115)	3.273	0.010
8.5 : 1 – 4.6 : 1	-0.080 (0.106)	-0.751	0.944
1.6 : 1 – 4.6 : 1	0.055 (0.109)	0.508	0.987
8.5 : 1 – 2.5 : 1	-0.457 (0.108)	-4.233	< 0.001
1.6 : 1 – 2.5 : 1	-0.322 (0.111)	-2.907	0.030
1.6 : 1 – 8.5 : 1	0.135 (0.101)	1.331	0.671

Table S2.3 Output from post hoc Tukey analysis of model exploring the effect of diet on the final length of fish.

Comparison	Estimate. (s.e.)	z	*p*
4.6 : 1 – 10.2 : 1	0.225 (1.594)	0.141	> 0.999
2.5 : 1 – 10.2 : 1	3.292 (1.615)	2.038	0.247
8.5 : 1 – 10.2 : 1	-2.399 (1.484)	-1.617	0.486
1.6 : 1 – 10.2 : 1	-0.120 (1.523)	-0.079	> 0.999
2.5 : 1 – 4.6 : 1	3.067 (1.649)	1.860	0.338
8.5 : 1 – 4.6 : 1	-2.625 (1.519)	-1.727	0.416
1.6 : 1 – 4.6 : 1	-0.347 (1.558)	-0.222	0.999
8.5 : 1 – 2.5 : 1	-5.692 (1.545)	-3.684	0.002
1.6 : 1 – 2.5 : 1	-3.413 (1.583)	-2.155	0.196
1.6 : 1 – 8.5 : 1	2.279 (1.452)	1.570	0.516

Table S2.4 Output from post hoc Tukey analysis of model exploring the effect of diet on carcass dry weight.

Comparison	Estimate. (s.e.)	z	*p*
4.6 : 1 – 10.2 : 1	-0.022 (0.037)	-0.585	0.977
2.5 : 1 – 10.2 : 1	0.133 (0.038)	3.515	0.004
8.5 : 1 – 10.2 : 1	-0.066 (0.035)	-1.886	0.324
1.6 : 1 – 10.2 : 1	0.006 (0.036)	0.153	> 0.999
2.5 : 1 – 4.6 : 1	0.155 (0.038)	4.053	< 0.001
8.5 : 1 – 4.6 : 1	-0.045 (0.036)	-1.251	0.721
1.6 : 1 – 4.6 : 1	0.027 (0.037)	0.752	0.944
8.5 : 1 – 2.5 : 1	-0.120 (0.036)	-5.519	< 0.001
1.6 : 1 – 2.5 : 1	-0.128 (0.037)	-3.442	0.005
1.6 : 1 – 8.5 : 1	0.072 (0.034)	-2.091	0.224

Table S2.5 Output from post hoc Tukey analysis of model exploring the effect of diet on condition index.

Comparison	Estimate. (s.e.)	z	*p*
4.6 : 1 – 10.2 : 1	-0.096 (0.045)	-1.779	0.207
2.5 : 1 – 10.2 : 1	0.075 (0.046)	1.640	0.482
8.5 : 1 – 10.2 : 1	0.040 (0.042)	0.938	0.881
1.6 : 1 – 10.2 : 1	0.024 (0.043)	0.469	0.981
2.5 : 1 – 4.6 : 1	0.171 (0.047)	3.303	0.003
8.5 : 1 – 4.6 : 1	0.136 (0.043)	2.765	0.015
1.6 : 1 – 4.6 : 1	0.120 (0.044)	2.274	0.051
8.5 : 1 – 2.5 : 1	-0.035 (0.044)	-0.808	0.934
1.6 : 1 – 2.5 : 1	-0.050 (0.045)	-1.225	0.796
1.6 : 1 – 8.5 : 1	-0.016 (0.041)	-0.472	0.996

Table S2.6 Output from post hoc Tukey analysis of model exploring the effect of diet on the ratio of Protein : Lipid in the carcass.

Comparison	Estimate. (s.e.)	z	*p*
4.6 : 1 – 10.2 : 1	-0.692 (0.377)	-1.835	0.353
2.5 : 1 – 10.2 : 1	-1.653 (0.382)	-4.320	< 0.001
8.5 : 1 – 10.2 : 1	0.697 (0.358)	1.946	0.292
1.6 : 1 – 10.2 : 1	-1.251 (0.365)	-3.432	0.005
2.5 : 1 – 4.6 : 1	-0.961 (0.387)	-2.481	0.095
8.5 : 1 – 4.6 : 1	1.389 (0.363)	3.830	0.001
1.6 : 1 – 4.6 : 1	-0.560 (0.369)	-1.515	0.552
8.5 : 1 – 2.5 : 1	2.350 (0.370)	6.354	< 0.001
1.6 : 1 – 2.5 : 1	0.402 (0.375)	1.071	0.821
1.6 : 1 – 8.5 : 1	-1.949 (0.350)	-5.567	< 0.001

Table S2.7 Output from post hoc Tukey analysis of model exploring the effect of diet on the difference in Protein : Lipid content between diet and carcass, i.e. degree of change in Protein : Lipid.

Comparison	Estimate. (s.e.)	z	*p*
4.6 : 1 – 10.2 : 1	4.978 (0.377)	13.190	< 0.001
2.5 : 1 – 10.2 : 1	6.061 (0.383)	15.825	< 0.001
8.5 : 1 – 10.2 : 1	2.412 (0.359)	6.735	< 0.001
1.6 : 1 – 10.2 : 1	7.355 (0.365)	20.154	< 0.001
2.5 : 1 – 4.6 : 1	1.083 (0.388)	2.792	0.041
8.5 : 1 – 4.6 : 1	-2.563 (0.363)	-7.058	< 0.001
1.6 : 1 – 4.6 : 1	2.377 (0.370)	6.430	< 0.001
8.5 : 1 – 2.5 : 1	-3.645 (0.370)	-9.845	< 0.001
1.6 : 1 – 2.5 : 1	1.294 (0.375)	3.448	0.005
1.6 : 1 – 8.5 : 1	4.939 (0.350)	14.097	< 0.001

Table S2.8 Output from post hoc Tukey analysis of model exploring the effect of diet on protein content of carcass, with dry weight included in the model.

Comparison	Estimate. (s.e.)	z	*p*
4.6 : 1 – 10.2 : 1	-0.017 (0.003)	-4.394	< 0.001
2.5 : 1 – 10.2 : 1	-0.033 (0.004)	-8.066	< 0.001
8.5 : 1 – 10.2 : 1	-0.004 (0.004)	-1.207	0.747
1.6 : 1 – 10.2 : 1	-0.020 (0.004)	-5.502	< 0.001
2.5 : 1 – 4.6 : 1	-0.016 (0.004)	-3.851	0.001
8.5 : 1 – 4.6 : 1	0.012 (0.004)	3.344	0.007
1.6 : 1 – 4.6 : 1	-0.003 (0.004)	-0.930	0.885
8.5 : 1 – 2.5 : 1	0.028 (0.004)	6.822	< 0.001
1.6 : 1 – 2.5 : 1	0.013 (0.004)	3.167	0.013
1.6 : 1 – 8.5 : 1	-0.016 (0.004)	-4.408	< 0.001

Table S2.9 Output from post hoc Tukey analysis of model exploring the effect of diet on lipid content of carcass, with dry weight included in the model.

Comparison	Estimate. (s.e.)	z	*p*
4.6 : 1 – 10.2 : 1	0.013 (0.005)	2.396	0.117
2.5 : 1 – 10.2 : 1	0.034 (0.006)	6.057	< 0.001
8.5 : 1 – 10.2 : 1	-0.000 (0.005)	-0.033	1.000
1.6 : 1 – 10.2 : 1	0.023 (0.005)	4.588	< 0.001
2.5 : 1 – 4.6 : 1	0.021 (0.006)	3.719	0.002
8.5 : 1 – 4.6 : 1	-0.013 (0.005)	-2.530	0.084
1.6 : 1 – 4.6 : 1	0.011 (0.005)	2.070	0.232
8.5 : 1 – 2.5 : 1	-0.034 (0.006)	-6.017	< 0.001
1.6 : 1 – 2.5 : 1	-0.011 (0.005)	-1.952	0.289
1.6 : 1 – 8.5 : 1	0.023 (0.005)	4.783	< 0.001

Table S2.10 Output from censored exponential MCMCglmm model. All results are non-significant.

	Posterior mean	Lower 95% CI	Upper 95% CI	Effective sample size	pMCMC
Intercept	225.540	-193.348	663.043	1000	0.342
8.5:1	65.665	-45.209	207.681	1000	0.314
4.6:1	41.635	-82.533	180.794	1000	0.542
2.5:1	51.654	-66.268	173.139	1000	0.386
1.6:1	113.842	-10.893	229.301	1000	0.080
Weight	-1.771	-9.194	6.871	1000	0.654
Sex (male)	-18.969	-97.846	50.116	1000	0.628
Temperature	-24.466	-69.437	19.413	1000	0.300

nitt = 1,300,000; thin = 1,000; burnin = 300,000

Table S2.11 Estimate (s.e.) from analysis of activity (total time active).

	Estimate (s.e.)
Intercept	17.203 (3.687)
8.5 : 1	1.337 (2.671)
4.6 : 1	0.191 (2.532)
2.5 : 1	0.037 (2.560)
1.6 : 1	3.696 (2.577)
Sex (male)	-1.387 (1.746)
Weight	-3.166 (3.559)

S2.2 Supplementary Figures

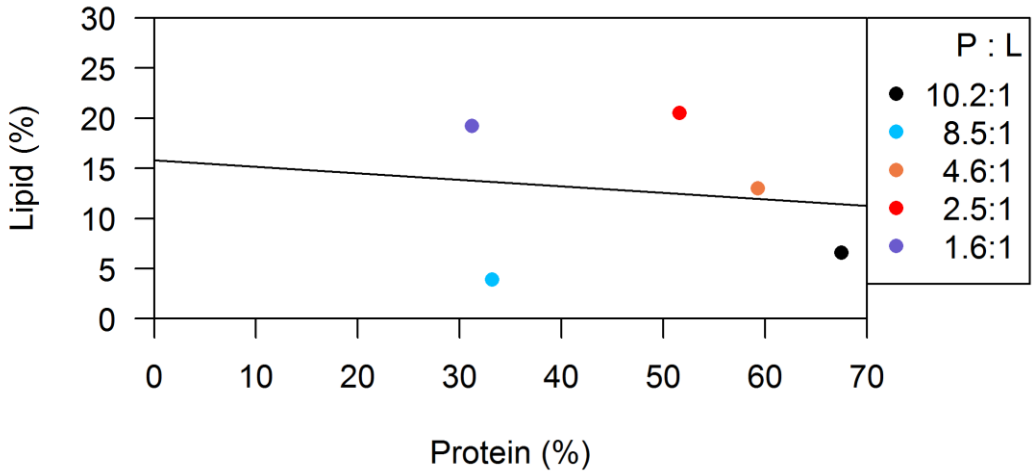

Figure S2.1 The relationship between the lipid (%) and protein (%) contents of the five diets. Colours indicate the diet (see key). The black line represents the regression line from a linear model of lipid content against protein content (slope = -0.0649±0.264). Pearson's correlation analysis shows protein and lipid are not strongly negatively correlated in the diets (Pearson's correlation = -0.141 (95% confidence interval = -0.910 to 0.847), t_3 = -0.246, p = 0.822).

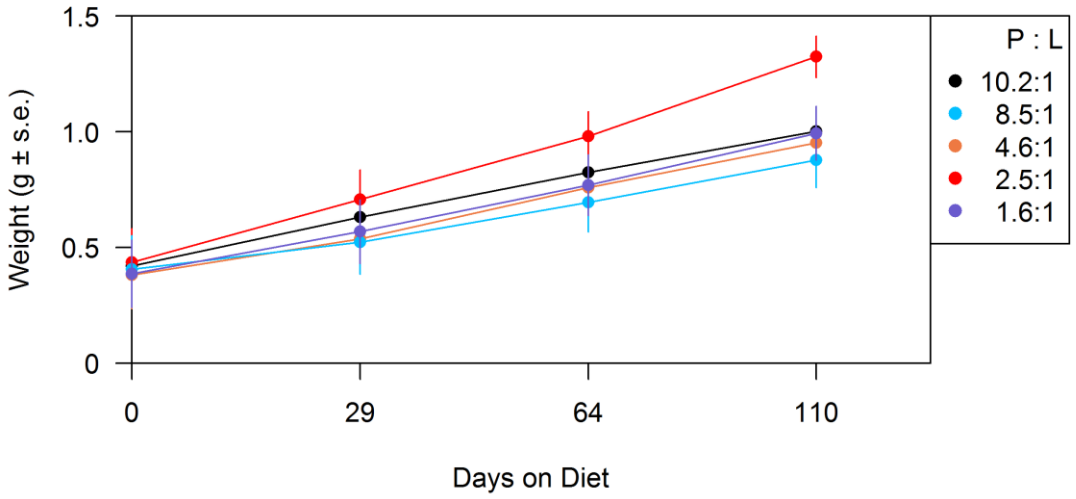

Figure S2.2 Mean weight (± s.e.) in relation to the number of days on the diet treatments. Colours indicate diets (see key). There was no difference between diet treatments initially (p = 0.716). However there was a significant effect of diet on final weight (p = 0.001), where *2.5:1* diet is significantly different from all other diets (all $p < 0.040$).

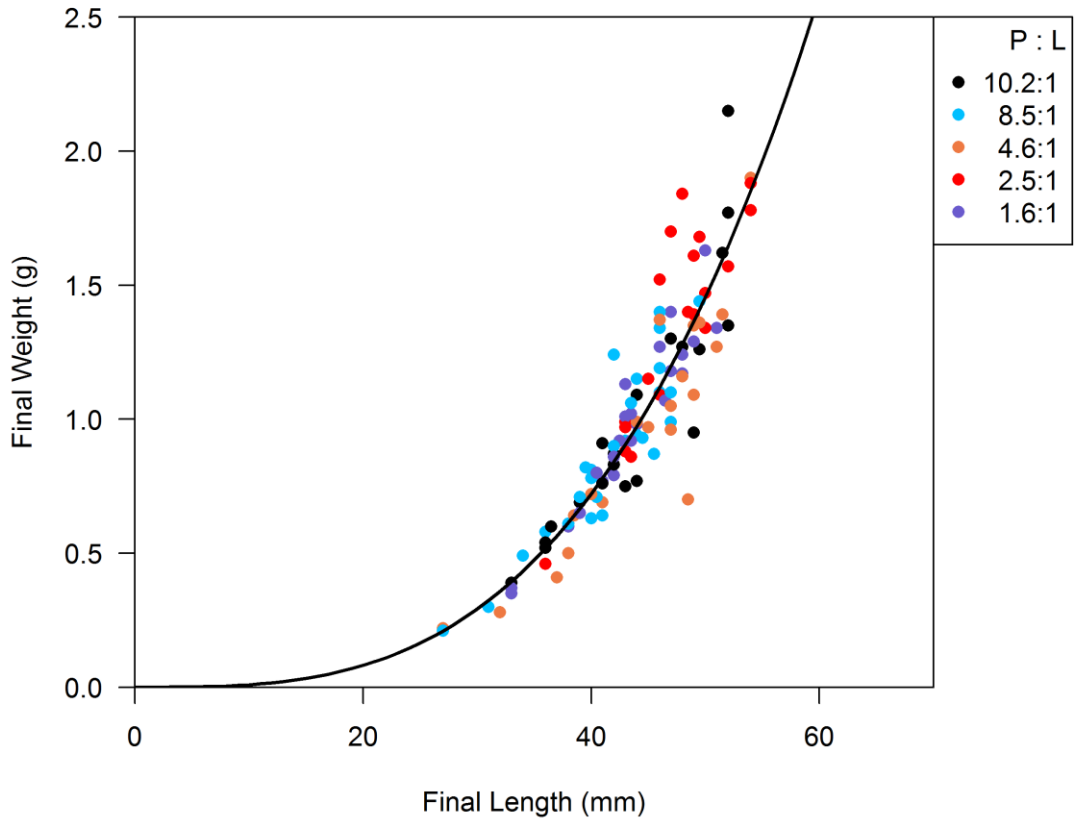

Figure S2.3 Final length of fish against final weight of fish. You can see the expected non-linear relationship between weight and length. Colours correspond to the five diets (see key). The black line represents the predicted weight for fish, calculated as above. Points above this line have a positive condition index, points below have a negative condition index.

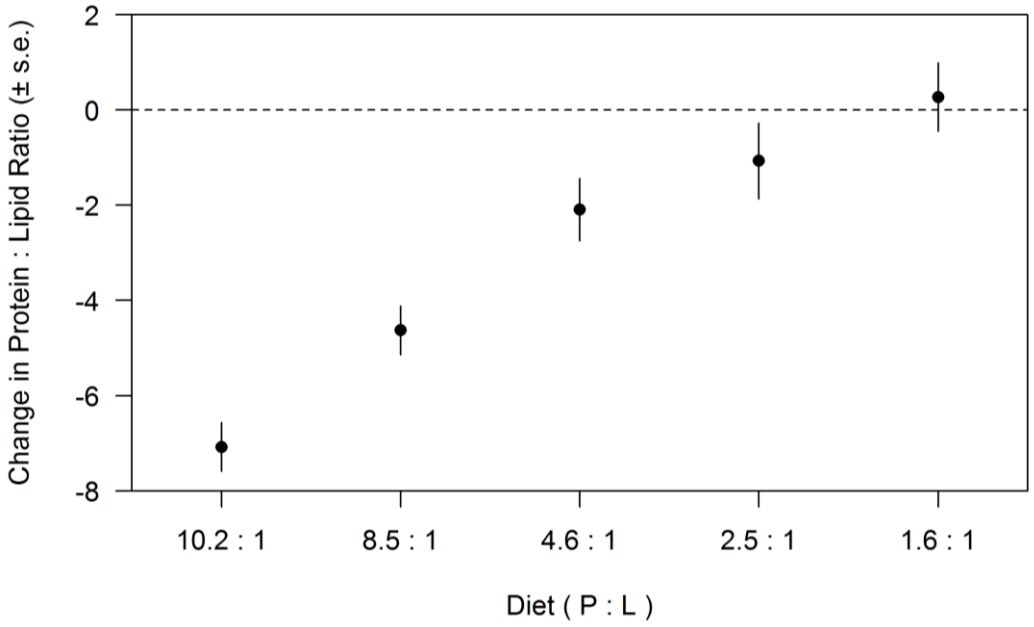

Figure S2.4 Mean difference between the dietary protein : lipid ratio and carcass protein : lipid ratio (± s.e.). The dashed line represents zero, or no difference in the protein : lipid ratio of the diet compared to that of the carcass. The change in protein : lipid was significantly different between all the diets (all $p < 0.041$).

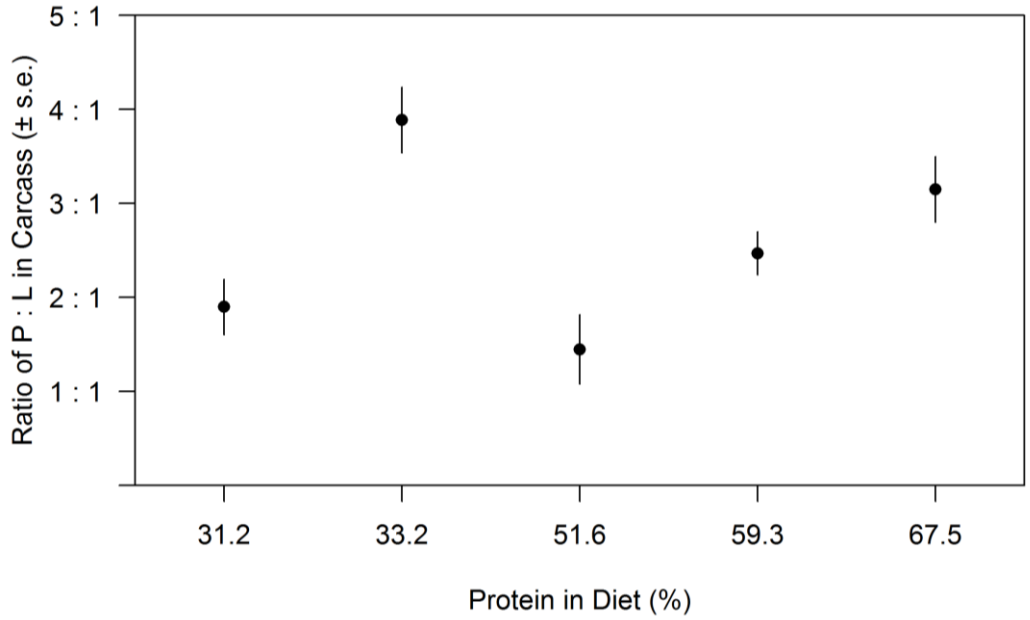

Figure S2.5 Mean (± s.e.) carcass protein : lipid ratio in relation to dietary protein (%).Ratio in carcass is carcass protein (g) / carcass lipid (g). Ratio of protein to lipid in the carcass decreased linearly with increasing dietary lipid intake ($p < 0.001$), but is not significantly affected by protein intake ($p = 0.180$).

Appendix 3:

Sex-specific effects of nutrient intake on mortality risk but not reproduction.

S3.1 Supplementary methods

Measurement of food intakes

To restrict the amount of food eaten as well as varying macronutrient content, individual fish were fed a specific ration across the experiment (Terzibasi et al., 2009). To calculate this ration we used monthly monitoring of sentinel fish. Each month, we identified two individuals for each sex*diet*size combination from the 100% feeding level (e.g. 100% fed large male on the 10.2:1 diet, see supplementary table S1). Food was added to the tank in small increments and feeding behaviour was observed. If all the food was consumed another portion of food was added to the tank. This continued until satiation. This was done at both feeding periods for a single day. We then took an average of the amount of food eaten across the four feeding assessments (two individuals with two assessments each), and this ration was fed to all individuals on that treatment for the next month. The individuals used to determine food ration were the two median sized fish of that treatment.

Fish showing signs of ill health

For welfare reasons and to comply with home office regulations in the UK, any individuals showing signs of ill health were monitored closely and, if symptoms persisted for two consecutive days, were humanely sacrificed via overdose of tricaine mesylate (MS222) and physical destruction of the brain. Symptoms of ill health were typically gulping at the surface or bottom of the tank, inability to maintain an upright

position whilst swimming or females being egg bound. Fish showing these symptoms typically do not recover (Walling personal observation) and so we do not feel this protocol biased on data on survival in any way.

Reproduction Second Breeding Season

As so few individuals survived to and reproduced during the second breeding season (N alive = 242), it was not possible to look at the same reproductive measures as breeding season one. We therefore analysed whether an individual attempted to reproduce or not (attempted = 1, not attempted = 0; conditional on having survived to the second breeding season) using a general linear mixed model (GLME) with binomial error distribution. Here, sex was fitted as a categorical fixed effect and protein and lipid included as continuous covariates. To account for whether individuals survived throughout the breeding season, we included survival as a categorical fixed effect (y = survived, n = died during the second breeding season) as well as the proportion of individuals alive at the start of the second breeding season (one proportion for each diet*level*sex*size combination). For this analysis amounts of protein and lipid were quantified as the total amount eaten (g) up to the start of the second breeding season and then z-transformed.

There was a positive linear effect of lipid on reproductive status (GLME; $\chi^2 = 5.41$; $p = 0.020$) and a significant effect of survival status, with those individuals surviving the whole breeding season being more likely to attempt reproduction (GLME; $\chi^2 = 10.34$; $p = 0.001$). However, there was no effect of protein intake (GLME; $\chi^2 = 0.16$; $p = 0.689$), sex (GLME; $\chi^2 = 0.38$; $p = 0.540$) or proportion of

individuals alive at the start of the breeding season (GLME; $\chi^2 = 0.04$; $p = 0.840$) on reproductive status.

Measurement of nuptial colouration

Male breeding colour was assessed via monthly photographs using a standard procedure for sticklebacks (Frischknecht, Braithwaite and Barber, 2000, Barber et al., 2001). Briefly, fish were removed from their home tank and immediately placed in a glass-sided photographing chamber filled with water. Males were placed so their left side faced towards the camera and were temporarily fixed in place with a piece of damp sponge. The photographing chamber was then placed onto a small viewing stage and an image taken under standard light conditions (see below). Immediately after the picture was taken, males' were returned to their home tank. The whole process from removal to returning a male its home tank took approximately 60s and was designed to minimise the stress experienced by the males. This procedure was performed approximately once a month from the start of the breeding season (taken as when 20% of males were expressing nuptial colour) and ceased when the breeding season was deemed to have ended (when less than 20% of males were displaying colour). Light conditions where standardised by the use of two lamps containing broad spectrum daylight bulbs angled at 45^o towards the viewing stage, with no flash used on the camera. Photographs were taken with a Pentax K_r digital camera (F2.8, shutter speed 1/125), fitted with a Tamron 90mm macro lens fixed in position directly in front of the viewing stage. The relative positions of the viewing stage, camera and two lamps remained constant throughout the experiment. All photographs were taken alongside a scale bar and white, grey and black colour standards.

Photographs were analysed using the software ImageJ. Briefly, the white standard was analysed to give standard values for red, green and blue light. The fish was then highlighted using the polygon selection tool ensuring just the main body of the fish was included. The intensity and area of red colouration were recorded and the process was then repeated for blue colour. Prior to statistical analysis all measures were standardised by dividing them by the colour values measured from the white colour standard.

Nesting

During the experiment, male nesting was assessed. Once a male was deemed to have come into breeding condition (when they began expressing nuptial colour), they were provided with standard nesting material, consisting of approximately 200 6cm long black cotton threads and sand (Barber et al., 2001). Males were then stimulated to build nests by presenting them with an image of a sexually mature (gravid) female for 5 minutes, once per day. Nests were checked daily and the time until nest construction started and time until construction was completed were recorded. Males were given 2 weeks at each of these stages of nest construction (i.e. two weeks to start a nest, 2 weeks to complete a nest and 2 weeks with a completed nest), at the end of the two week period the nesting material was removed and replaced with fresh material. Nesting assessment was continued until a male failed to attempt nest construction on three successive occasions, after which no further nesting material was provided.

Territory Defence and Courtship Behavioural Assays

Alongside nesting ability, males were assayed for their investment in courtship and territory defence. During the breeding season, males display aggressive behaviour towards any red object (Tinbergen, 1951). Therefore, we stimulated male aggressive behaviour by suspending a red pen lid in the male's home tank. Males were exposed to the red object once per nest (within 7 days of construction beginning) for a period of 5 minutes. During this time we recorded: reaction time, total time displaying aggressive behaviour, number of aggressive swims and number of biting attempts. For final analysis we explored: the average reaction time, total time displaying aggressive behaviour and the total number of aggressive swims across all trials for each individual male.

Similarly, male courtship can be stimulated by exposing the male to an image of a sexually mature female (JPM and CAW personal observation). Male courtship behaviour comprises a number of discrete steps (see Wootton, 1984), the two most recognisable steps being the 'zig-zag dance' towards the female, during which the male rapidly swims from side to side, followed by the male 'leading' the female to the nest and swimming through the entrance (Wootton, 1984). We assayed courtship ability 7 days after a nest was judged to be completed. As with the territory defence assay, males were assessed for 5 minutes, during which an image of a gravid female was attached to the front of the tank. During this time we recorded: reaction time, total time spent courting the female, number of zig-zag dances and number of leads. For final analysis we analysed the average reaction time, total time spent courting, total number of zigzag dances and the total number of leads across all trials for each individual male.

Egg Stripping

All females were checked every other day to see if they were gravid (indicated by a swollen and distended abdomen (Barber and Arnott, 2000)). Gravid females were removed from the tank, quickly dried, weighed and the eggs stripped and placed into a petri dish. Egg stripping involved gently running a finger down the side of the fish towards the tail, which encouraged the expulsion of the egg sack. This was repeated on both sides of the fish, ensuring all eggs were expelled. The fish was then quickly reweighed and returned to their original tank. The whole process from removal to returning to home tank took approximately 60s. The eggs were then spread into a monolayer using fine forceps and a paintbrush and counted twice to ensure accuracy. The number of eggs was taken as the average between the two numbers (rounded to the nearest whole number). Clutch mass was calculated as the difference in pre- and post- stripping weight of the female. Fish were stripped whenever they became gravid with the shortest interval between two egg strips being 3 days.

S3.2 Supplementary Tables

Table S3.1 Number of individuals in each diet treatment. Initially 10 individuals of each sex and size class (L = Large, S = Small) were assigned to each treatment. However, due to mortality immediately prior to the experiment and some errors in molecular sexing (N=10), the final sample sizes were as below.

Diet	Sex	Size	100%	75%	50%	Total
1	F	L	9	10	10	29
		S	10	9	10	29
	M	L	11	10	10	31
		S	10	10	10	30
2	F	L	10	10	10	30
		S	10	10	10	30
	M	L	10	10	9	29
		S	10	9	10	29
3	F	L	12	10	11	33
		S	10	10	10	30
	M	L	8	10	9	27
		S	9	10	10	29
4	F	L	11	10	10	31
		S	9	9	9	27
	M	L	9	10	10	29
		S	11	10	11	32
5	F	L	9	10	9	28
		S	10	10	10	30
	M	L	11	10	10	31
		S	10	10	10	30
		Total	199	197	198	**594**

Table S3.2 Table showing the content (%) of different ingredients in the five diets used in this experiment. Diets are described by their protein : lipid ratio (P:L). Herring meal is both a source of protein and lipid, therefore fish oil was only required in diets with high lipid contents.

	Diet (P:L)				
Ingredient	**10.2 : 1**	**8.5 : 1**	**4.6 : 1**	**2.5 : 1**	**1.6 : 1**
Herring Meal	90.24	41.65	79.83	69.42	41.65
Corn Starch (Filler)	6.26	54.85	11.17	13.61	38.77
Lecithin	1.00	1.00	1.00	1.00	1.00
Vitamin /mineral premix	1.00	1.00	1.00	1.00	1.00
ASTX (10% carophyll pink)	1.00	1.00	1.00	1.00	1.00
CMC binder	0.50	0.50	0.50	0.50	0.50
Fish Oil	0.00	0.00	5.50	13.47	16.08

Table S3.3 Outputs from event history models exploring Male mortality risk. Models are binomial generalised linear mixed models (GLME). Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	χ^2	***p***
intercept	-3.999 (0.349)		
Time Period 2	-1.076 (0.299)		
Time Period 3	0.561 (0.319)		
Time Period 4	-0.334 (0.356)		
Time Period 5	1.813 (0.399)		
Time Period 6	1.639 (0.520)	89.15	< 0.001
Protein	0.223 (0.133)	2.87	0.090
Lipid	-0.396 (0.136)	9.47	0.002
Initial Weight	-1.044 (0.597)	3.29	0.070
Protein^2	1.095 (0.738)	2.20	0.138
Lipid^2	1.379 (0.657)	4.32	0.038
Protein*Lipid	-0.321 (0.295)	1.17	0.279

Table S3.4 Outputs from event history models (binomial GLME) exploring Female mortality risk. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	χ^2	***p***
intercept	-5.040 (0.408)		
Time Period 2	0.034 (0.341)		
Time Period 3	1.736 (0.381)		
Time Period 4	1.185 (0.444)		
Time Period 5	3.058 (0.597)		
Time Period 6	3.333 (0.819)	78.38	< 0.001
Protein	0.032 (0.094)	0.12	0.733
Lipid	-0.002 (0.089)	< 0.00	0.981
Initial Weight	-0.066 (0.499)	0.02	0.894
Protein^2	-0.243 (0.435)	0.31	0.576
Lipid^2	0.255 (0.453)	0.31	0.575
Protein*Lipid	-0.032 (0.160)	0.04	0.843

Table S3.5 Outputs from linear mixed models (LME) exploring the linear and non-linear effects of protein and lipid on various measures of male courtship effort.

	Estimate (± s.e.)	χ^2	***p***
Reaction Time			
Protein	-10.63 (6.59)	2.61	0.106
Lipid	11.63 (6.79)	2.94	0.087
Protein^2	9.80 (42.74)	0.05	0.817
Lipid^2	25.93 (36.94)	0.04	0.483
Protein * Lipid	5.42 (14.04)	0.15	0.700
ZigZag dances			
Protein	4.39 (1.81)	5.86	0.015
Lipid	-1.55 (1.84)	0.73	0.393
Protein^2	-6.42 (11.66)	0.31	0.576
Lipid^2	-15.09 (10.11)	2.12	0.146
Protein * Lipid	3.72 (3.88)	0.93	0.335
Leads			
Protein	1.56 (0.74)	4.47	0.034
Lipid	-0.14 (0.75)	0.04	0.842
Protein^2	-4.67 (4.76)	0.99	0.321
Lipid^2	-6.67 (4.13)	2.61	0.106
Protein * Lipid	1.96 (1.58)	1.56	0.212

Table S3.6 Outputs from LME models exploring the linear and non-linear effects of protein and lipid on various measures of male territory defence.

	Estimate (± s.e.)	χ^2	***p***
Aggressive displays			
Protein	1.03 (0.55)	3.48	0.062
Lipid	-1.26 (0.56)	5.08	0.024
Protein^2	-0.08 (3.61)	0.0002	0.988
Lipid^2	-1.97 (3.09)	0.040	0.527
Protein * Lipid	0.89 (1.19)	0.55	0.457
Reaction Time			
Protein	-15.70 (5.59)	7.67	0.006
Lipid	9.90 (5.77)	2.92	0.088
Protein^2	-22.64 (36.56)	0.39	0.535
Lipid^2	-11.71 (31.28)	0.14	0.707
Protein * Lipid	-3.05 (11.93)	0.07	0.793

Table S3.7. Outputs from LME models exploring the linear and non-linear effects of protein and lipid on various measures of male nesting behaviour.

	Estimate (± s.e.)	χ^2	***p***
Nesting Attempts			
Protein	-0.123 (0.240)	0.30	0.586
Lipid	0.424 (0.244)	3.02	0.082
Protein^2	-0.143 (1.561)	0.01	0.918
Lipid^2	-2.003 (1.334)	2.29	0.130
Protein * Lipid	-0.122 (0.522)	0.05	0.818
Completed Nests			
Protein	0.116 (0.172)	0.47	0.493
Lipid	0.257 (0.173)	2.16	0.141
Protein^2	0.098 (1.123)	< 0.00	0.998
Lipid^2	-0.940 (0.965)	0.01	0.922
Protein * Lipid	0.004 (0.378)	0.90	0.343

Table S3.8 Outputs from LME models exploring the linear and non-linear effects of protein and lipid on various measures of female egg production.

	Estimate (± s.e.)	χ^2	*p*
Mean Egg Number			
Protein	4.895 (1.907)	6.56	0.010
Lipid	9.236 (1.897)	22.91	< 0.001
Protein^2	-10.860 (11.154)	0.973	0.324
Lipid^2	-46.642 (10.523)	18.91	< 0.001
Protein * Lipid	7.828 (3.579)	4.75	0.029
Number of Clutches			
Protein	0.739 (0.350)	4.22	0.040
Lipid	0.088 (0.349)	0.07	0.789
Protein^2	0.596 (2.113)	0.09	0.769
Lipid^2	-4.135 (1.991)	4.37	0.036
Protein * Lipid	0.231 (0.678)	0.12	0.731
Total Egg Mass			
Protein	0.67 (0.17)	14.47	< 0.001
Lipid	0.11 (0.17)	0.44	0.508
Protein^2	-0.20 (1.02)	0.04	0.846
Lipid^2	-3.19 (0.96)	11.02	< 0.001
Protein * Lipid	0.47 (0.33)	2.14	0.144
Clutch Interval			
Protein	-1.05 (0.48)	4.40	0.036
Lipid	0.23 (0.48)	0.21	0.648
Protein^2	3.22 (2.93)	1.16	0.282
Lipid^2	9.24 (2.72)	11.43	< 0.001
Protein * Lipid	-2.45 (0.93)	6.92	0.008

Table S3.9 Outputs from LME models of reproductive senescence in male courtship (time spent courting (s)). All models included the non-linear effect of Age and the *p* value for Age was estimated using the package lmerTest as it is only significant when Age^2 was included in the model. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	χ^2	***p***
intercept	-0.039 (0.072)		
Age	1.114 (0.226)		< 0.001
Age^2	-1.088 (0.226)	22.95	< 0.001
Age First	-0.131 (0.043)	9.39	0.002
Age Last	0.069 (0.043)	2.60	0.107
Protein	0.118 (0.049)	5.65	0.017
Lipid	-0.082 (0.050)	2.78	0.096
$Age\ First^2$	0.577 (0.297)	3.83	0.050
$Age\ Last^2$	-0.021 (0.444)	0.00	0.946
$Protein^2$	-0.284 (0.317)	0.86	0.353
$Lipid^2$	-0.266 (0.268)	1.02	0.312
Protein*Lipid	0.107 (0.102)	1.19	0.275

Table S3.10 Outputs from LME models exploring the linear and non-linear effects of age, protein and lipid on male nuptial colour (red intensity). All models included the non-linear effect of Age and the *p* value for Age was estimated using the package lmerTest as it is only significant when Age^2 was included in the model. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	χ^2	***p***
intercept	0.477 (0.005)		
Age	0.256 (0.009)		< 0.001
Age^2	-0.258 (0.009)	593.06	< 0.001
Age Last	0.003 (0.002)	3.24	0.071
Protein	0.002 (0.002)	0.61	0.435
Lipid	0.005 (0.003)	3.95	0.047
$Age\ Last^2$	0.014 (0.023)	0.37	0.544
$Protein^2$	-0.024 (0.015)	2.57	0.109
$Lipid^2$	-0.038 (0.013)	8.40	0.004
Protein*Lipid	0.012 (0.005)	5.49	0.019

Table S3.11 Outputs from LME models of reproductive senescence in female egg production (number of eggs laid). All models included the non-linear effect of Age and the *p* value for Age was estimated using the package lmerTest as it is only significant when Age^2 was included in the model. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	χ^2	***p***
Intercept	-0.042 (0.059)		
Age	1.389 (0.121)	108.99	< 0.001
Age^2	-1.560 (0.120)	161.86	< 0.001
Age First	-0.128 (0.043)	9.00	0.002
Age Last	0.036 (0.034)	1.14	0.285
Protein	0.100 (0.054)	3.42	0.064
Lipid	0.266 (0.053)	24.57	< 0.001
$Age\ First^2$	0.054 (0.306)	0.03	0.858
$Age\ Last^2$	0.780 (0.323)	5.75	0.016
$Protein^2$	-0.273 (0.285)	0.96	0.327
$Lipid^2$	-1.225 (0.281)	18.50	< 0.001
Protein*Lipid	0.206 (0.097)	4.59	0.032

S3.3 Supplementary Figures

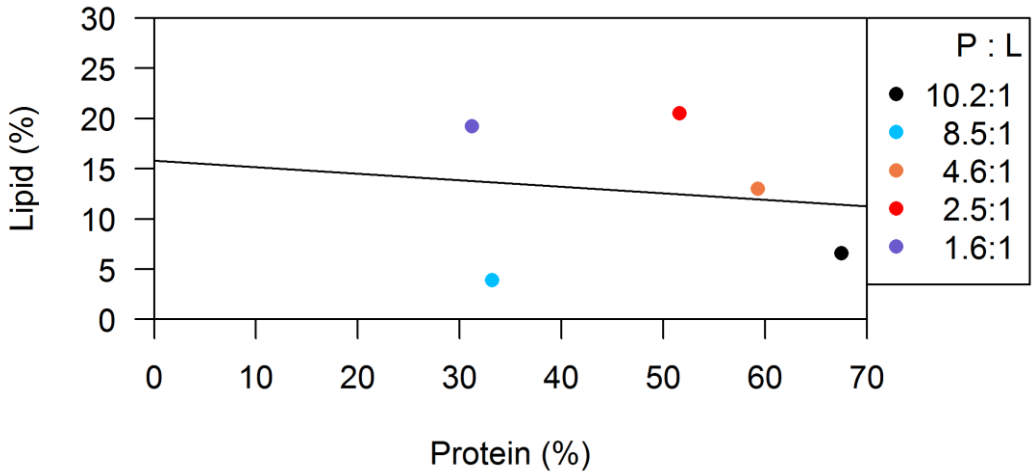

Figure S3.1 The relationship between the lipid (%) and protein (%) contents of the five diets. Colours indicate the diet (see key). The black line represents the regression line from a linear model of lipid content against protein content (slope = -0.0649±0.264). Pearson's correlation analysis shows protein and lipid are not strongly negatively correlated in the diets (Pearson's correlation = -0.141 (95% confidence interval = -0.910 to 0.847), t_3 = -0.246, p = 0.822).

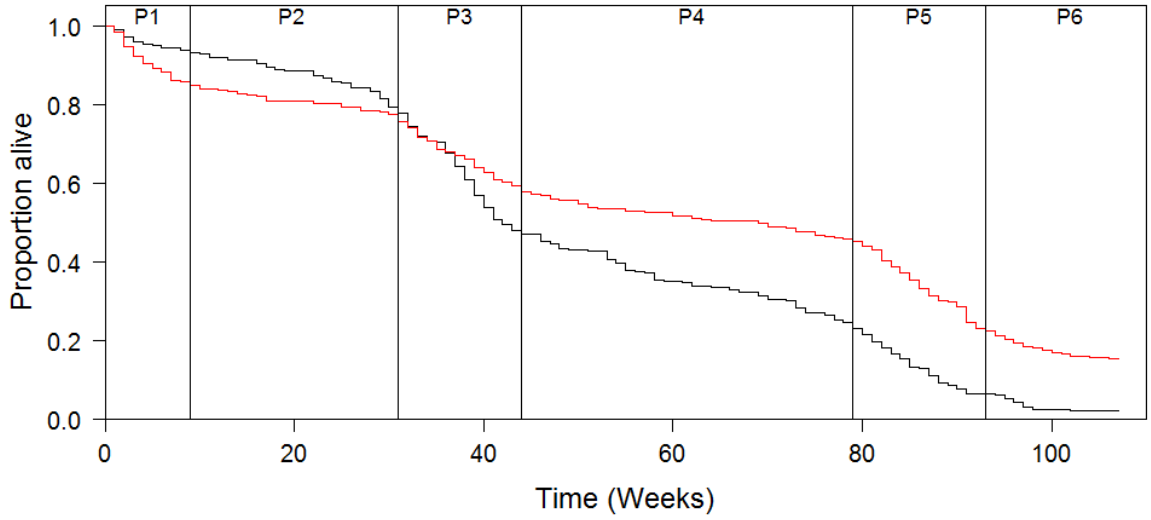

Figure S3.2. Kaplan-Meier Survival plot showing the relationship between time (weeks) and proportion of individuals alive. The 6 time periods that were included in the survival models are represented as P1 – P6. The black line represents females and the red line males.

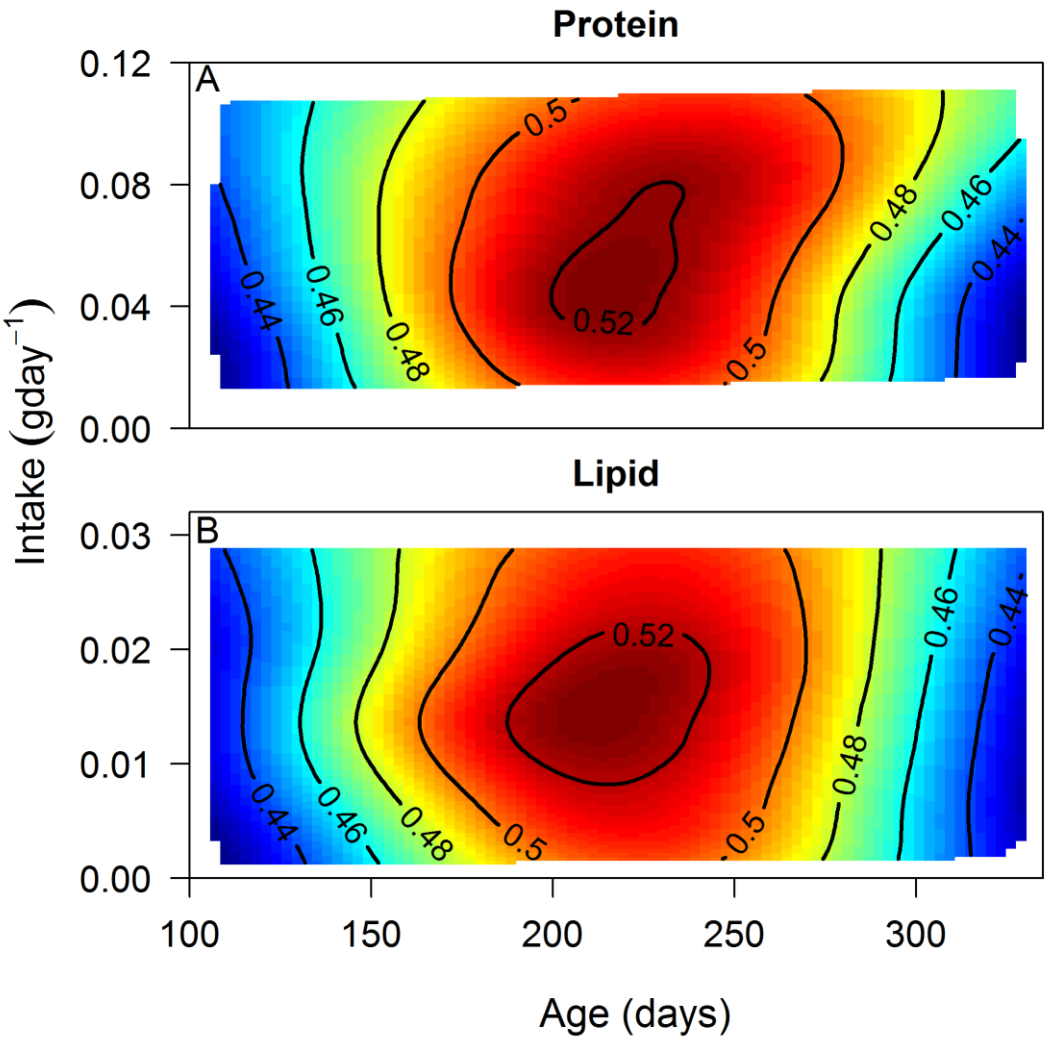

Figure S3.3. Non-parametric thin-plate spline contour plots showing the effect of age (days) and macronutrient intake on male breeding colour (red intensity) across the first breeding season. Panel (A) protein intake (gday^{-1}), panel (B) lipid intake (gday^{-1}).

Appendix 4:

The effect of diet on growth, condition and swimming performance

S4.1 Supplementary Tables

Table S4.1 Outputs from models of the effect of macronutrient intake on male weight. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	0.697 (0.021)	1949	1, 431.7	< 0.001
Batch 2	0 (NA)			
Batch 3	0.340 (0.012)			
Batch 4	0.888 (0.023)			
Batch 5	1.022 (0.027)			
Batch 6	1.144 (0.033)			
Batch 7	1.261 (0.032)			
Batch 8	1.454 (0.036)			
Batch 9	1.895 (0.043)			
Batch 10	2.168 (0.043)	404.50	8, 763.6	< 0.001
Protein	-0.061 (0.022)	7.99	1, 824.5	0.005
Lipid	0.073 (0.022)	10.68	1, 667.2	0.001
Protein^2	-0.393 (0.093)	17.88	1, 1364.8	< 0.001
Lipid^2	-0.334 (0.080)	17.39	1, 1193.0	< 0.001
Protein*Lipid	0.181 (0.039)	21.52	1, 1128.7	< 0.001

Table S4.2 Outputs from model of the effect of macronutrient intake on male weight. Model contains main effects that were significant in previous models (Appendix 4: Table S4.1) and their interactions. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***P***
(Intercept)	0.980 (0.170)	2129	1, 485.8	< 0.001
Batch 2	0 (NA)			
Batch 3	-0.107 (0.130)			
Batch 4	0.461 (0.157)			
Batch 5	0.609 (0.162)			
Batch 6	0.742 (0.165)			
Batch 7	0.822 (0.166)			
Batch 8	1.013 (0.168)			
Batch 9	1.444 (0.172)			
Batch 10	1.249 (0.320)	476.60	8, 756.6	< 0.001
Protein	-0.282 (0.285)	4.02	1, 1176.6	0.045
Lipid	0.081 (0.214)	17.34	1, 742.9	< 0.001
Protein^2	0.799 (0.567)	6.75	1, 964.3	0.010
Lipid^2	-0.034 (0.238)	23.59	1, 1065.7	< 0.001
Protein*Lipid	0.025 (0.129)	13.27	1, 942.8	< 0.001
Batch 2*Protein	0 (NA)			
Batch 3*Protein	0.569 (0.184)			
Batch 4*Protein	0.544 (0.232)			
Batch 5*Protein	0.461 (0.250)			
Batch 6*Protein	0.388 (0.271)			
Batch 7*Protein	0.509 (0.272)			
Batch 8*Protein	0.508 (0.283)			
Batch 9*Protein	0.465 (0.301)			
Batch 10*Protein	1.173 (0.604)	0.65	8, 860.7	0.739
Batch 2*Lipid	0 (NA)			
Batch 3*Lipid	0.576 (0.130)			
Batch 4*Lipid	0.623 (0.171)			
Batch 5*Lipid	0.651 (0.188)			
Batch 6*Lipid	0.686 (0.212)			
Batch 7*Lipid	0.715 (0.210)			

Table S4.2 continued

	Estimate (± s.e.)	F	d.f. (1,2)	*p*
Batch 8*Lipid	0.711 (0.223)			
Batch 9*Lipid	0.789 (0.244)			
Batch 10*Lipid	2.181 (0.532)	7.73	8, 832.2	< 0.001
Batch 2*Protein^2	0 (NA)			
Batch 3*Protein^2	-1.296 (0.401)			
Batch 4*Protein^2	-1.285 (0.499)			
Batch 5*Protein^2	-1.172 (0.523)			
Batch 6*Protein^2	-1.087(0.540)			
Batch 7*Protein^2	-1.168 (0.546)			
Batch 8*Protein^2	-1.142 (0.555)			
Batch 9*Protein^2	-1.096 (0.567)			
Batch 10*Protein^2	-2.333 (1.052)	2.41	8, 848.1	0.014
Batch 2*Lipid^2	0 (NA)			
Batch 3*Lipid^2	-0.596 (0.148)			
Batch 4*Lipid^2	-0.593 (0.186)			
Batch 5*Lipid^2	-0.544 (0.204)			
Batch 6*Lipid^2	-0.556 (0.229)			
Batch 7*Lipid^2	-0.571 (0.229)			
Batch 8*Lipid^2	-0.553 (0.241)			
Batch 9*Lipid^2	-0.556 (0.261)			
Batch 10*Lipid^2	-2.586 (0.675)	5.19	8, 837.3	< 0.001
Batch 2*Protein*Lipid	0 (NA)			
Batch 3*Protein*Lipid	0.205 (0.079)			
Batch 4*Protein*Lipid	0.198 (0.103)			
Batch 5*Protein*Lipid	0.152 (0.111)			
Batch 6*Protein*Lipid	0.119 (0.119)			
Batch 7*Protein*Lipid	0.129 (0.121)			
Batch 8*Protein*Lipid	0.112 (0.126)			
Batch 9*Protein*Lipid	0.084 (0.134)			
Batch 10*Protein*Lipid	0.357 (0.294)	2.07	8, 821	0.036

Table S4.3 Outputs from models of the effect of macronutrient intake on male length. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	38.840 (0.304)	59890	1, 343.3	< 0.001
Batch 2	0 (NA)			
Batch 3	4.513 (0.166)			
Batch 4	9.150 (0.255)			
Batch 5	10.484 (0.298)			
Batch 6	10.445 (0.363)			
Batch 7	12.026 (0.355)			
Batch 8	13.462 (0.376)			
Batch 9	17.805 (0.425)			
Batch 10	17.849 (0.385)	334.80	8, 844.1	< 0.001
Protein	-0.727 (0.193)	14.06	1, 660.4	< 0.001
Lipid	1.035 (0.203)	25.86	1, 541.9	< 0.001
Protein^2	-0.393 (0.092)	5.30	1, 742.3	0.022
Lipid^2	-0.333 (0.080)	25.35	1, 686.3	< 0.001
Protein*Lipid	0.180 (0.038)	8.54	1, 621.6	0.004

Table S4.4 Outputs from model of the effect of macronutrient intake on male length. Model contains main effects that were significant in previous models (Appendix 4: Table S4.3) and their interactions. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(intercept)	37.399 (0.639)	28060.00	1, 580.8	< 0.001
Batch 2	0 (NA)			
Batch 3	5.265 (0.408)			
Batch 4	9.990 (0.551)			
Batch 5	11.228 (0.576)			
Batch 6	11.024 (0.614)			
Batch 7	12.651 (0.599)			
Batch 8	14.115 (0.612)			
Batch 9	18.322 (0.646)			
Batch 10	19.336 (0.734)	320.10	8, 854.9	< 0.001
Protein	0.839 (0.968)	1.35	1, 1075.1	0.246
Lipid	3.143 (0.871)	40.90	1, 766.0	< 0.001
Protein^2	-2.159 (0.995)	4.70	1, 856.4	0.030
Lipid^2	-3.857 (0.833)	21.43	1, 887.2	< 0.001
Protein*Lipid	0.873 (0.370)	5.55	1, 816.0	0.019
Batch 2*Protein	0 (NA)			
Batch 3*Protein	0.122 (0.494)			
Batch 4*Protein	-0.129 (0.656)			
Batch 5*Protein	0.076 (0.717)			
Batch 6*Protein	0.345 (0.761)			
Batch 7*Protein	0.097 (0.783)			
Batch 8*Protein	0.417 (0.800)			
Batch 9*Protein	0.343 (0.825)			
Batch 10*Protein	0.346 (0.972)	1.08	8, 822.0	0.376
Batch 2*Lipid	0 (NA)			
Batch 3*Lipid	1.299 (0.346)			
Batch 4*Lipid	1.553 (0.488)			
Batch 5*Lipid	1.952 (0.541)			
Batch 6*Lipid	2.025 (0.583)			
Batch 7*Lipid	2.163 (0.587)			
Batch 8*Lipid	1.797 (0.607)			
Batch 9*Lipid	2.167 (0.642)			
Batch 10*Lipid	2.667 (0.790)	3.68	8, 820.7	< 0.001

Table S4.5 Outputs from models of the effect of macronutrient intake on female weight. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	0.796 (0.020)	2728	1, 545.9	< 0.001
Batch 2	0 (NA)			
Batch 3	0.334 (0.010)			
Batch 4	1.073 (0.027)			
Batch 5	1.329 (0.032)			
Batch 6	1.417 (0.041)			
Batch 7	1.590 (0.043)			
Batch 8	1.920 (0.053)			
Batch 9	2.630 (0.074)			
Batch 10	3.187 (0.087)	319.20	8, 564.4	< 0.001
Protein	0.039 (0.023)	2.84	1, 756.5	0.093
Lipid	0.031 (0.023)	1.78	1, 567.1	0.183
Protein^2	-0.091 (0.093)	0.75	8, 647.2	0.651
Lipid^2	-0.263 (0.123)	2.43	8, 637.2	0.014
Protein*Lipid	0.127 (0.045)	1.18	8, 642.8	0.306

Table S4.6 Outputs from model of the effect of macronutrient intake on female weight. Model contains main effects that were significant in previous models (Appendix 4: Table S4.5) Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	0.682 (0.045)	1242	1, 606.6	< 0.001
Batch 2	0 (NA)			
Batch 3	0.351 (0.024)			
Batch 4	1.085 (0.036)			
Batch 5	1.31 (0.038)			
Batch 6	1.383 (0.048)			
Batch 7	1.545 (0.052)			
Batch 8	1.86 (0.061)			
Batch 9	2.539 (0.082)			
Batch 10	3.213 (0.255)	350.90	8, 552.8	< 0.001
Protein	0.251 (0.081)	0.38	1, 690.2	0.539
Lipid	0.343 (0.11)	6.89	1, 763.6	0.009
Protein^2	-0.242 (0.096)	6.33	1, 798.1	0.012
Lipid^2	-0.381 (0.163)	21.03	1, 814.5	< 0.001
Protein*Lipid	0.196 (0.048)	16.44	1, 672.9	< 0.001
Batch 2*Lipid	0 (NA)			
Batch 3*Lipid	0.151 (0.057)			
Batch 4*Lipid	0.212 (0.085)			
Batch 5*Lipid	0.399 (0.086)			
Batch 6*Lipid	0.289 (0.118)			
Batch 7*Lipid	0.408 (0.111)			
Batch 8*Lipid	0.497 (0.139)			
Batch 9*Lipid	0.639 (0.195)			
Batch 10*Lipid	1.318 (0.534)	3.66	8, 597.7	< 0.001
Batch 2*Lipid^2	0 (NA)			
Batch 3*Lipid^2	-0.151 (0.106)			
Batch 4*Lipid^2	-0.156 (0.125)			
Batch 5*Lipid^2	-0.329 (0.126)			
Batch 6*Lipid^2	-0.243 (0.147)			
Batch 7*Lipid^2	-0.354 (0.139)			
Batch 8*Lipid^2	-0.405 (0.156)			
Batch 9*Lipid^2	-0.467 (0.194)			
Batch 10*Lipid^2	-1.365 (1.058)	1.59	8, 633.2	0.126

Table S4.7 Outputs from models of the effect of macronutrient intake on female length. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	40.461 (0.300)	58920	1, 384.4	< 0.001
Batch 2	0 (NA)			
Batch 3	4.733 (0.163)			
Batch 4	10.99 (0.254)			
Batch 5	14.143 (0.317)			
Batch 6	14.496 (0.412)			
Batch 7	16.348 (0.601)			
Batch 8	17.405 (0.613)			
Batch 9	23.077 (0.678)			
Batch 10	25.805 (0.581)	429.70	8, 604.7	< 0.001
Protein	0.207 (0.270)	0.59	1, 525.6	0.444
Lipid	1.090 (0.260)	17.52	1, 457.3	< 0.001
Protein^2	-0.527 (1.055)	0.25	1, 956.1	0.617
Lipid^2	-4.589 (1.218)	14.19	1, 728.0	< 0.001
Protein*Lipid	1.085 (0.451)	5.78	1, 701.0	0.016

Table S4.8 Outputs from model of the effect of macronutrient intake on female length. Model contains main effects that were significant in previous models (Appendix 4: Table S4.7). Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	39.115 (0.402)	41100.00	1, 541.7	< 0.001
Batch 2	0 (NA)			
Batch 3	5.301 (0.216)			
Batch 4	11.941 (0.316)			
Batch 5	15.107 (0.363)			
Batch 6	15.231 (0.447)			
Batch 7	17.094 (0.647)			
Batch 8	18.242 (0.642)			
Batch 9	23.681 (0.707)			
Batch 10	27.306 (0.744)	431.70	8, 601.8	< 0.001
Protein	0.152 (0.276)	1.421	1, 520.5	0.234
Lipid	3.546 (0.872)	20.20	1, 757.4	< 0.001
Lipid^2	-4.841 (0.982)	24.27	1, 862.4	< 0.001
Protein*Lipid	0.677 (0.265)	6.51	1, 992.6	0.011
Batch 2*Lipid	0 (NA)			
Batch 3*Lipid	0.866 (0.274)			
Batch 4*Lipid	1.53 (0.381)			
Batch 5*Lipid	1.927 (0.445)			
Batch 6*Lipid	2.254 (0.53)			
Batch 7*Lipid	2.323 (0.678)			
Batch 8*Lipid	2.014 (0.675)			
Batch 9*Lipid	2.608 (0.721)			
Batch 10*Lipid	3.081 (1.022)	3.15	8, 609.2	0.002

Table S4.9 Outputs from models of the effect of macronutrient intake on male condition index. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	0.022 (0.009)	10.09	1, 459.1	< 0.001
Batch 2	0 (NA)			
Batch 3	0.003 (0.008)			
Batch 4	-0.012 (0.011)			
Batch 5	0.002 (0.012)			
Batch 6	0.033 (0.017)			
Batch 7	-0.079 (0.016)			
Batch 8	-0.031 (0.014)			
Batch 9	-0.048 (0.015)			
Batch 10	0.010 (0.013)	11.56	8, 678.7	< 0.001
Protein	-0.003 (0.006)	0.19	1, 462.8	0.667
Lipid	0.041 (0.006)	38.61	1, 439.9	< 0.001
$Protein^2$	-0.038 (0.035)	1.15	1, 533.0	0.285
$Lipid^2$	-0.056 (0.030)	3.52	1, 421.9	0.061
Protein*Lipid	0.021 (0.014)	2.18	1, 389.7	0.140

Table S4.10 Outputs from model of the effect of macronutrient intake on male condition index. Model contains main effects that were significant in previous models (Appendix 4: Table S4.9). Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	0.107 (0.073)	< 0.00	1, 449.0	0.959
Batch 2	0 (NA)			
Batch 3	-0.110 (0.067)			
Batch 4	-0.094 (0.073)			
Batch 5	-0.085 (0.073)			
Batch 6	-0.050 (0.074)			
Batch 7	-0.209 (0.074)			
Batch 8	-0.136 (0.074)			
Batch 9	-0.145 (0.074)			
Batch 10	-0.294 (0.095)	12.43	8, 668.8	< 0.001
Protein	-0.046 (0.102)	0.08	1, 559.3	0.774
Lipid	0.070 (0.056)	30.62	1, 517.9	< 0.001
Protein^2	0.254 (0.256)	0.01	1, 569.0	0.922
Lipid^2	-0.098 (0.102)	10.87	1, 544.3	0.001
Batch 2*Protein	0 (NA)			
Batch 3*Protein	0.090 (0.096)			
Batch 4*Protein	0.023 (0.103)			
Batch 5*Protein	0.048 (0.104)			
Batch 6*Protein	-0.017 (0.115)			
Batch 7*Protein	0.130 (0.111)			
Batch 8*Protein	0.078 (0.108)			
Batch 9*Protein	0.076 (0.110)			
Batch 10*Protein	0.310 (0.151)	1.81	8, 633.9	0.072
Batch 2*Lipid	0 (NA)			
Batch 3*Lipid	0.036 (0.051)			
Batch 4*Lipid	-0.001 (0.058)			
Batch 5*Lipid	0.035 (0.060)			
Batch 6*Lipid	0.127 (0.077)			
Batch 7*Lipid	0.263 (0.074)			
Batch 8*Lipid	0.134 (0.067)			
Batch 9*Lipid	0.064 (0.072)			
Batch 10*Lipid	0.298 (0.100)	4.51	8, 623.6	< 0.001

Table S4.10 continued.

	Estimate (± s.e.)	F	d.f. (1,2)	*p*
Batch 2*Protein^2	0 (NA)			
Batch 3* Protein^2	-0.334 (0.231)			
Batch 4* Protein^2	-0.218 (0.251)			
Batch 5* Protein^2	-0.258 (0.255)			
Batch 6* Protein^2	-0.209 (0.259)			
Batch 7* Protein^2	-0.328 (0.258)			
Batch 8* Protein^2	-0.282 (0.257)			
Batch 9* Protein^2	-0.286 (0.258)			
Batch 10* Protein^2	-0.774 (0.330)	2.00	8, 641.7	0.045
Batch 2*Lipid^2	0 (NA)			
Batch 3*Lipid^2	-0.012 (0.091)			
Batch 4*Lipid^2	0.051 (0.100)			
Batch 5*Lipid^2	0.036 (0.102)			
Batch 6*Lipid^2	-0.048 (0.112)			
Batch 7*Lipid^2	-0.131 (0.111)			
Batch 8*Lipid^2	-0.04 (0.107)			
Batch 9*Lipid^2	0.019 (0.110)			
Batch 10*Lipid^2	-0.437 (0.182)	3.65	8, 624.7	< 0.001

Table S4.11 Outputs from models of the effect of macronutrient intake on female condition index. Estimates (± s.e.) come from ASreml models, conditional F and *p* obtained through a Wald test. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Estimate (± s.e.)	**F**	**d.f. (1,2)**	***p***
(Intercept)	0.028 (0.009)	13.30	1, 499.2	< 0.001
Batch 2	0 (NA)			
Batch 3	-0.022 (0.008)			
Batch 4	-0.043 (0.011)			
Batch 5	-0.072 (0.011)			
Batch 6	-0.098 (0.016)			
Batch 7	0.001 (0.022)			
Batch 8	-0.059 (0.023)			
Batch 9	-0.041 (0.021)			
Batch 10	-0.036 (0.017)	7.83	8, 496.9	< 0.001
Protein	0.022 (0.007)	9.65	1, 510.2	0.002
Lipid	0.017 (0.006)	8.26	1, 488.8	0.004
$Protein^2$	-0.06 (0.034)	3.04	1, 711.6	0.082
$Lipid^2$	-0.018 (0.034)	0.29	1, 527.6	0.592
Protein*Lipid	0.012 (0.012)	0.89	1, 521.1	0.346

Table S4.12 The effect of protein and lipid intake on swimming endurance at trial 1. Model outputs are from MCMCglmm models (Poisson distribution, see Materials and Methods Chapter 5 for full details). Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Males			Females		
	Posterior Mean	95% CI	*p*	Posterior Mean	95% CI	*p*
(Intercept)	2.937	-3.486 to 9.625	0.360	-2.806	-9.948 to 3.738	0.442
Protein	0.113	-0.721 to 0.973	0.796	-0.747	-1.903 to 0.337	0.186
Lipid	-0.370	-1.310 to 0.518	0.424	-0.230	-1.253 to 0.896	0.618
Weight	-1.027	-2.96 to 0.732	0.268	1.047	-0.563 to 2.631	0.186
Water Temp	-0.144	-0.730 to 0.356	0.586	-0.121	-0.674 to 0.386	0.682
Protein^2	-1.393	-7.140 to 4.052	0.632	-3.584	-12.863 to 3.604	0.370
Lipid^2	-0.107	-4.675 to 4.468	0.964	-1.850	-7.005 to 3.488	0.474
Protein* Lipid	-0.010	-1.807 to 1.710	0.992	0.160	-2.035 to 2.206	0.884

nitt = 1,300,00; thin = 1,000; burnin= 300,000

Table S4.13 The effect of protein and lipid intake on swimming endurance at trial 2. Model outputs are from MCMCglmm models. Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Males			Females		
	Posterior Mean	95% CI	*p*	Posterior Mean	95% CI	*p*
(Intercept)	8.731	-45.875 to 63.972	0.782	-244.005	-1039.719 to 252.166	0.334
Protein	0.123	-1.701 to 1.978	0.882	-3.762	-28.306 to 14.640	0.682
Lipid	-0.890	-3.115 to 1.302	0.390	4.242	-18.087 to 33.545	0.660
Weight	-0.555	-4.907 to 3.551	0.814	-4.113	-47.952 to 26.241	0.780
Water Temp	-1.015	-4.813 to 2.331	0.594	11.447	-21.953 to 53.896	0.478
Protein^2	-1.532	-7.019 to 5.284	0.632			
Lipid^2	-0.103	-4.820 to 4.402	0.968			
Protein* Lipid	0.019	-1.868 to 1.782	0.994			

nitt = 1,300,00; thin = 1,000; burnin= 300,000

Table S4.14 Outputs for analysis of change in swim time between trials 1 and 2. Outputs are from linear mixed models (LME). Results presented below the line are taken from a model containing all linear terms and the listed non-linear and interaction effects, while those above come from a model containing only the linear terms.

	Males			Females		
	Estimate ± (s.e.)	χ^2	*p*	Estimate ± (s.e.)	χ^2	*p*
(Intercept)	-49.143 (23.085)			-54.857 (21.053)		
Protein	0.064 (26.009)	0.00	0.995	-8.794 (18.964)	0.17	0.676
Lipid	37.731 (26.025)	2.12	0.145	10.879 (18.911)	0.29	0.589
Protein^2	-97.860 (178.930)	0.31	0.580	172.890 (119.02)	2.18	0.140
Lipid^2	-63.400 (145.200)	0.20	0.655	136.620 (103.67)	1.81	0.178
Protein*Lipid	50.650 (59.130)	0.76	0.384	-11.510 (34.500)	0.12	0.729

Table S4.15 Outputs for analysis of sex differences in change in swim time between trials 1 and 2. Outputs are from linear mixed models (LME).

	Estimate ± (s.e.)	χ^2	*p*
(Intercept)	-59.030 (21.927)		
Protein	-0.935 (16.692)	< 0.00	0.957
Lipid	22.963 (16.694)	1.90	0.168
Sex (male)	9.635 (29.435)	0.12	0.729

S4.2 Supplementary Figures.

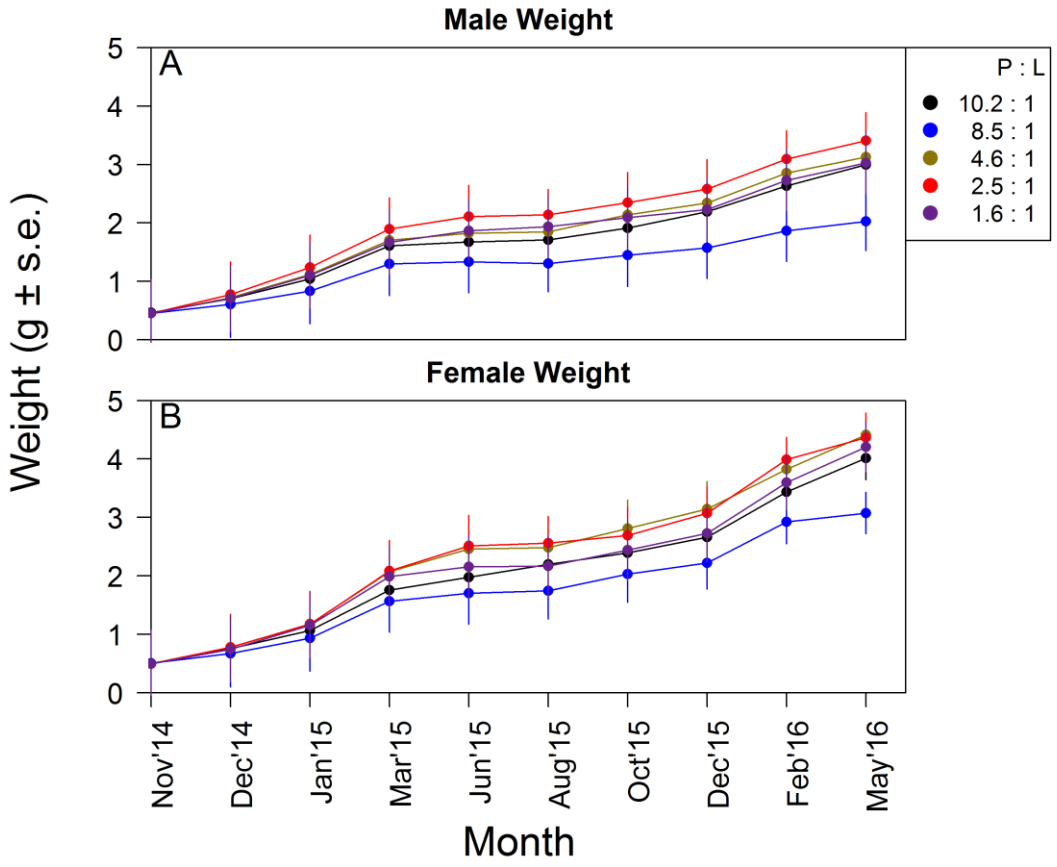

Figure S4.1 Mean weight (g ± s.e.) across weight batches. Coloured lines represent the five protein : lipid rations in the diets (see legend).

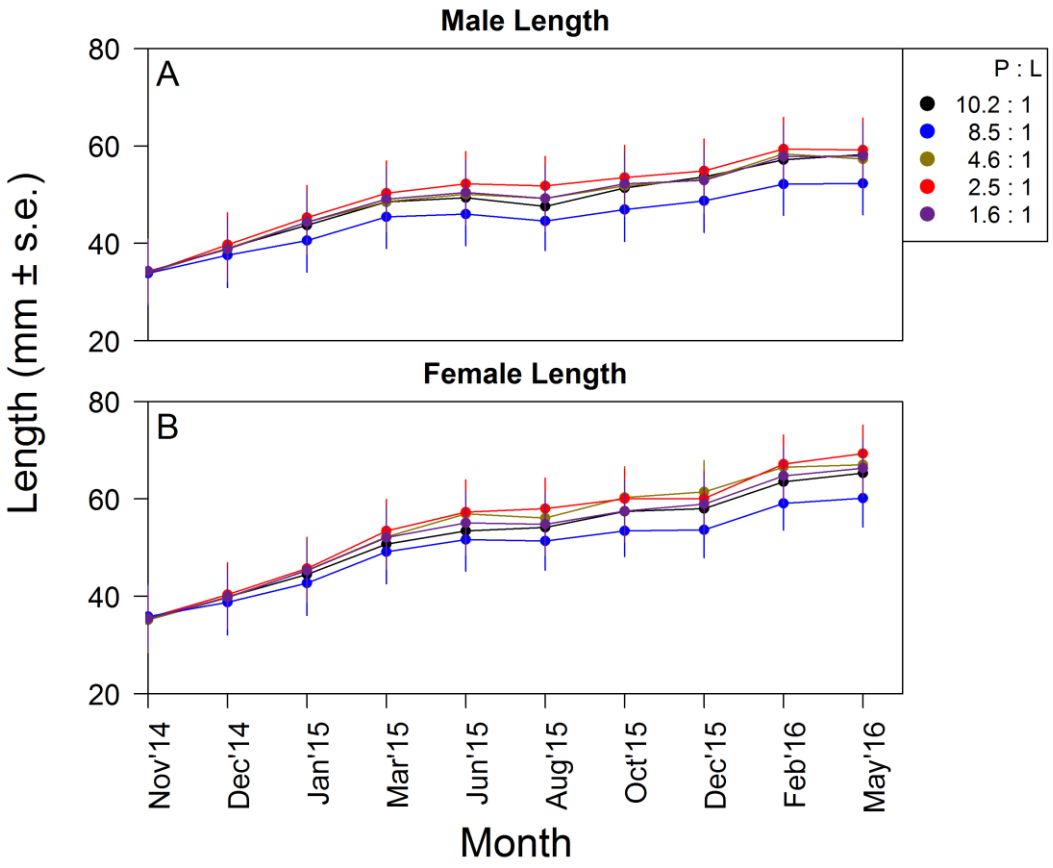

Figure S4.2. Mean length (mm ± s.e.) across weight batches. Coloured lines represent the five protein : lipid rations in the diets (see legend).

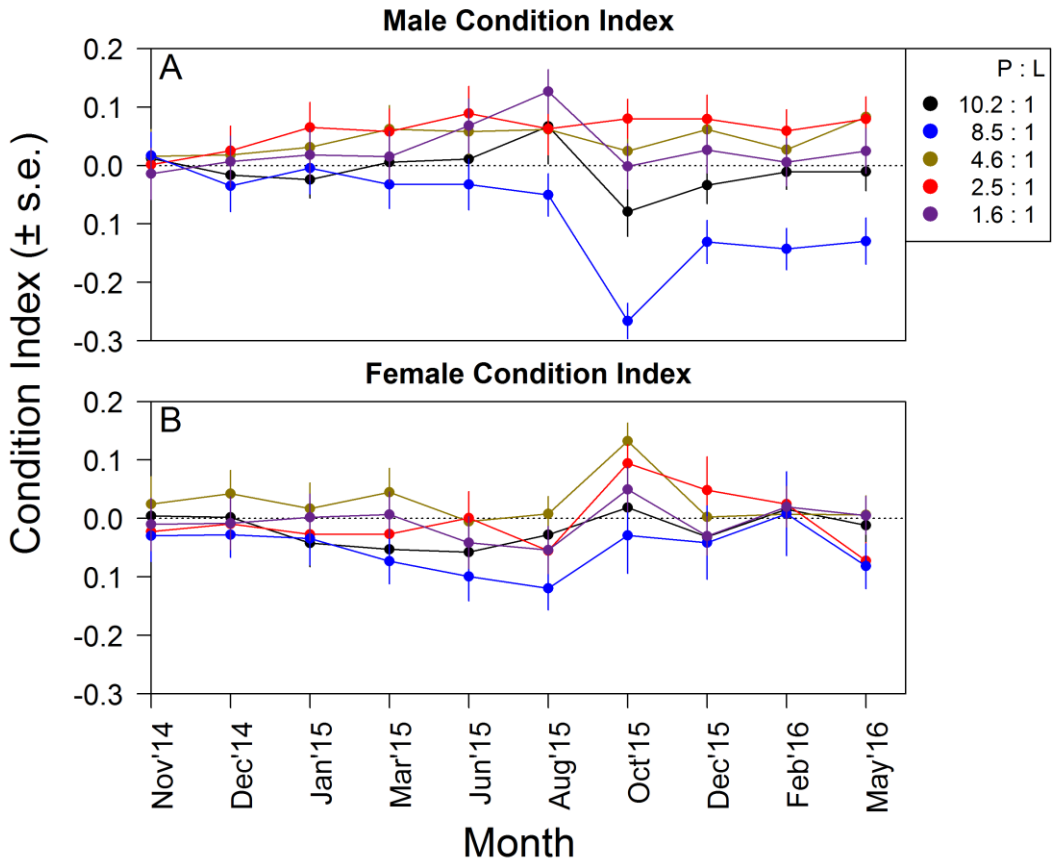

Figure S4.3. Mean condition index (± s.e.) across weight batches. Coloured lines represent the five protein : lipid rations in the diets (see legend). A positive value represents a better than average condition, negative lower than average, with 0 (dashed line) being average condition.

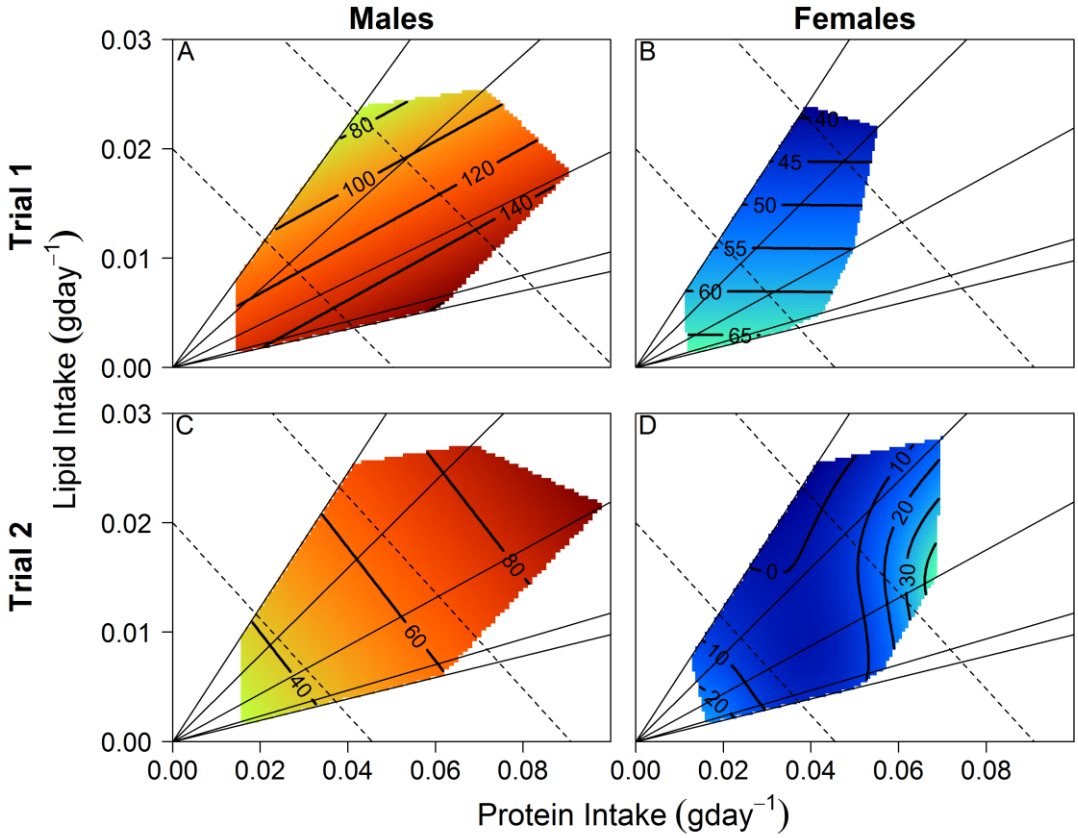

Figure S4.4 Non-parametric thin-plate spline contour plots showing the effect protein and lipid intake (gday^{-1}) on swimming endurance for Trial 1 (A & B) and Trial 2 (C & D). Panels (A) and (C) represent males, panels (B) and (D) represent females. Solid black lines coming from the origin represent the 5 diets used in this experiment, dashed lines represent isocaloric lines. There was no effect of macronutrient intake on swimming performance in either sex (all $p > 0.1$).

Appendix 5

Published Works

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BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

The effect of dietary restriction on reproduction: a meta-analytic perspective

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Abstract

Background: Dietary restriction (DR), a reduction in the amount of food or particular nutrients eaten, is the most consistent environmental manipulation to extend lifespan and protect against age related diseases. Current evolutionary theory explains this effect as a shift in the resolution of the trade-off between lifespan and reproduction. However, recent studies have questioned the role of reproduction in mediating the effect of DR on longevity and no study has quantitatively investigated the effect of DR on reproduction across species.

Results: Here we report a comprehensive comparative meta-analysis of the effect of DR on reproduction. In general, DR reduced reproduction across taxa, but several factors moderated this effect. The effect of DR on reproduction was greater in well-studied model species (yeast, nematode worms, fruit flies and rodents) than non-model species. This mirrors recent results for longevity and, for reproduction, seems to result from a faster rate of decline with decreasing resources in model species. Our results also suggested that not all reproductive traits are affected equally by DR. High and moderate cost reproductive traits suffered a significant reduction with DR, but low cost traits, such as ejaculate production, did not. Although the effect of DR on reproduction was stronger in females than males, this sex difference reduced to near zero when accounting for other co-factors such as the costliness of the reproductive trait. Thus, sex differences in the effect of DR on longevity may be due to a failure to expose males to as complete a range of the costs of reproduction as females.

Conclusions: We suggest that to better understand the generality of the effect of DR, future studies should attempt to address the cause of the apparent model species bias and ensure that individuals are exposed to as many of the costs of reproduction as possible. Furthermore, our meta-analytic approach reveals a general shortage of DR studies that record reproduction, particularly in males, as well as a lack of direct side-by-side comparisons of the effect of DR on males and females.

Keywords: Nutrition, Breeding, Life history trade-off, Meta-analysis, Systematic review

Background

Dietary restriction (DR), defined as a reduction in food intake without malnutrition [1, 2], has been shown to extend lifespan and protect against age related diseases across a range of studies (see [1, 3] for current reviews). The majority of studies examining DR use one of five laboratory model species: *Saccharomyces cerevisiae* [4], *Caenorhabditis elegans* [5], *Drosophila melanogaster* [6], *Mus musculus* and *Rattus norvegicus* [7], hereafter referred to as "model species" (see [1]). The taxonomic diversity of these model species and the fact that the effect of DR is reproducible in other, less commonly studied taxa (e.g. Primates [8]; arachnids [9]; fish [10]), has been used to suggest that the effect of DR on longevity is underpinned by an evolutionarily conserved mechanism and may thus have application to humans [3]. However, a recent meta-analysis has demonstrated that dietary restriction is nearly twice as effective at extending lifespan in the five model species as it is in non-model species [1]. Such an overarching pattern questions the taxonomic generality of this effect and thus the suggestion of an evolutionarily conserved mechanism.

The dominant evolutionary explanation of the effect of DR on longevity is based on the disposable soma theory of ageing [11, 12]. Under DR, it is hypothesised that

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organisms should reallocate resources away from reproduction to somatic maintenance (and thus survival) in order to increase the chance of surviving the period of resource limitation, and thus reproducing when more favourable conditions return [12]. A key prediction therefore is that increased longevity is a direct consequence of reduced reproduction. This prediction initially appears well supported; both among and within species fecundity is generally negatively correlated with longevity [13] and many studies cite a negative effect of DR on reproduction. However, close inspection reveals that these citations generally involve one of three studies: two using *D. melanogaster* [14, 15], cited 345 and 362 times respectively, (Google Scholar, accessed 07/09/2016) and the third study using rats [16], cited 89 times (Google Scholar, accessed 07/09/2016). More recently, studies have questioned the generality of the longevity-reproduction trade-off underlying the effect of DR, with some data suggesting that longevity and reproduction can be uncoupled [17, 18]. In *D. melanogaster*, for example, significant lifespan extension through DR was achieved in females that were incapable of vitellogenisis or had impaired ovarian activity and could not produce eggs [17]. Furthermore, many studies of DR fail to detect a decrease in reproduction, an increase in longevity or both [19–21]. These exceptions and the fact that a small number of studies using model species (where the DR effect on longevity is known to be greater [1]) are highly cited to support the longevity-reproduction trade-off underlying DR, suggest that an investigation into the generality of the effect of DR on reproduction is warranted.

One common observation is sexual dimorphism in the response to DR, with lifespan extension greater in females than in males [22–24]. Although direct comparisons between the sexes within the same study are rare (see below and [22]), the generality of this pattern has been supported by a recent meta-analysis showing a 20 % greater lifespan extension under DR in females than males [1]. An intuitive explanation is that females invest more in reproduction than males. However, although this may be true on a per-gamete basis, males invest heavily in reproduction via other avenues e.g. courtship, intra-male competition and territory defence, such that on average the net costs of reproduction must be equal in males and females [25, 26]. The fact that male costs of reproduction are generally not associated with gamete production may mean that males have not been exposed to the full costs of reproduction in current DR studies. In many studies males and females are kept separately and often in isolation (e.g. [21, 23, 27, 28]), and thus males do not experience the costs associated with e.g. courtship and competition. Thus, the sex difference in the effect of DR may be a result of sex differences in the costs of reproduction experienced. If this hypothesis is correct, we would predict a sex difference in the effect of DR on reproductive traits, with DR having more of an effect on higher cost traits. We expect that taking this into account will remove any sex difference in the effect of DR on reproduction.

Another area to explore is how reproductive decline changes with increasing levels of DR. The disposable soma theory of DR predicts an initially linear decrease in reproduction with decreasing resources. However, at very low levels of resources survival becomes unlikely and some degree of terminal investment is predicted [12], resulting in a decrease in the rate of reproductive decline. Recently an alternative to the disposable soma theory of DR has proposed that the response to DR evolved to minimise the loss of reproduction through upregulation of cell recycling mechanisms such as apoptosis and autophagy [29]. We suggest that this theory also predicts a non-linear reproductive decline with increasing DR. However, in this case the decrease in reproduction should be initially shallow, as cell recycling copes with small reductions in resources via recapture of some internal resources; a faster rate of decline should be observed at higher restriction levels. By examining the pattern of reproduction across levels of DR we can test these two hypotheses.

In this study we therefore attempt to address a number of issues surrounding the effect of DR on reproduction using a systematic review and meta-analysis. This method allows us to combine data from a diverse range of species, across a number of different studies. We can then highlight any general trends in the effect of DR on reproduction, whilst controlling for species-specific and study-specific effects. The specific aims of this paper are thus to investigate: (1) the generality of the effect of DR on reproduction; (2) whether, as for longevity, the effect of DR on reproduction is stronger in model than non-model species; (3) whether, as for longevity, there are sex differences in the effect of DR on reproduction; (4) whether these sex differences can be explained by the likely costliness of the reproductive traits investigated; and (5) the shape of reproductive decline with increasing restriction levels. More generally, this study aims to provide a quantitative summary of the current understanding of the effect of DR on reproduction and thus highlight areas where our knowledge is lacking and further research would be valuable.

Methods

Data collection and effect size extraction

Detailed descriptions of data collection and analysis are given in Additional file 1: Dialog S1. Briefly, data were collected through a search of *ISI Web of Science* and *Scopus* using the search strings 'diet*/calor* + restriction +

reproduction/fertility/fecundity'. Backward and forward searching was carried out to identify additional papers that were missed in the main database search and the authors' own literature collections on the subject were considered. These searches yielded 1679 papers (Fig. 1), of which 26 reported some measure of reproduction in treated (DR) and control females or males and matched the additional selection criteria (see Additional file 1: Dialog S1 for details). This is perhaps a surprisingly low number of studies given the interest in DR and longevity, highlighting the paucity of studies that also collect data on reproduction. Full details for why studies were rejected are provided in data S3 provided with our data supplement on dryad (doi:10.5061/dryad.3fc02), but a number of studies were rejected as a result of not applying DR consistently across life. It is worth noting that different selection criteria would result in a different selection of studies being included and may affect our results, but we do not think our selection criteria were overly restrictive or would cause any particular bias. The 26 studies used covered 21 species (see Additional file 1: Figure S1 for phylogenetic tree). From these 26 studies we extracted 205 effect sizes (based on 1096 control and 1132 treatment subjects), expressed as Cohen's *d*, calculated as:

$$d = \frac{\overline{x}_1 - \overline{x}_2}{s}$$

where $\overline{x}_1$ represents the mean value of the reproductive measure for the control group, $\overline{x}_2$ represents the mean for the treatment group and s represents the pooled standard deviation (for s calculation see Additional file 1: Dialog S1).

Moderators

In meta-analyses, the use of moderators (e.g. the effect of sex) is often required to explain variation in the effect across studies (heterogeneity [30], see Additional file 1: Dialog S1). Therefore, we extracted and examined the effect of the following moderators: (1) model species or not, (2) sex, (3) degree of restriction, (4) cost of reproductive trait (see below) and (5) type of control feeding (*Ad libitum* or 100 % feeding). As a result of the wide variety of reproductive measures taken, we attempted to categorise reproductive traits based on how much of the total cost of reproduction they were likely to represent. Reproductive traits were classified as: low cost, moderate

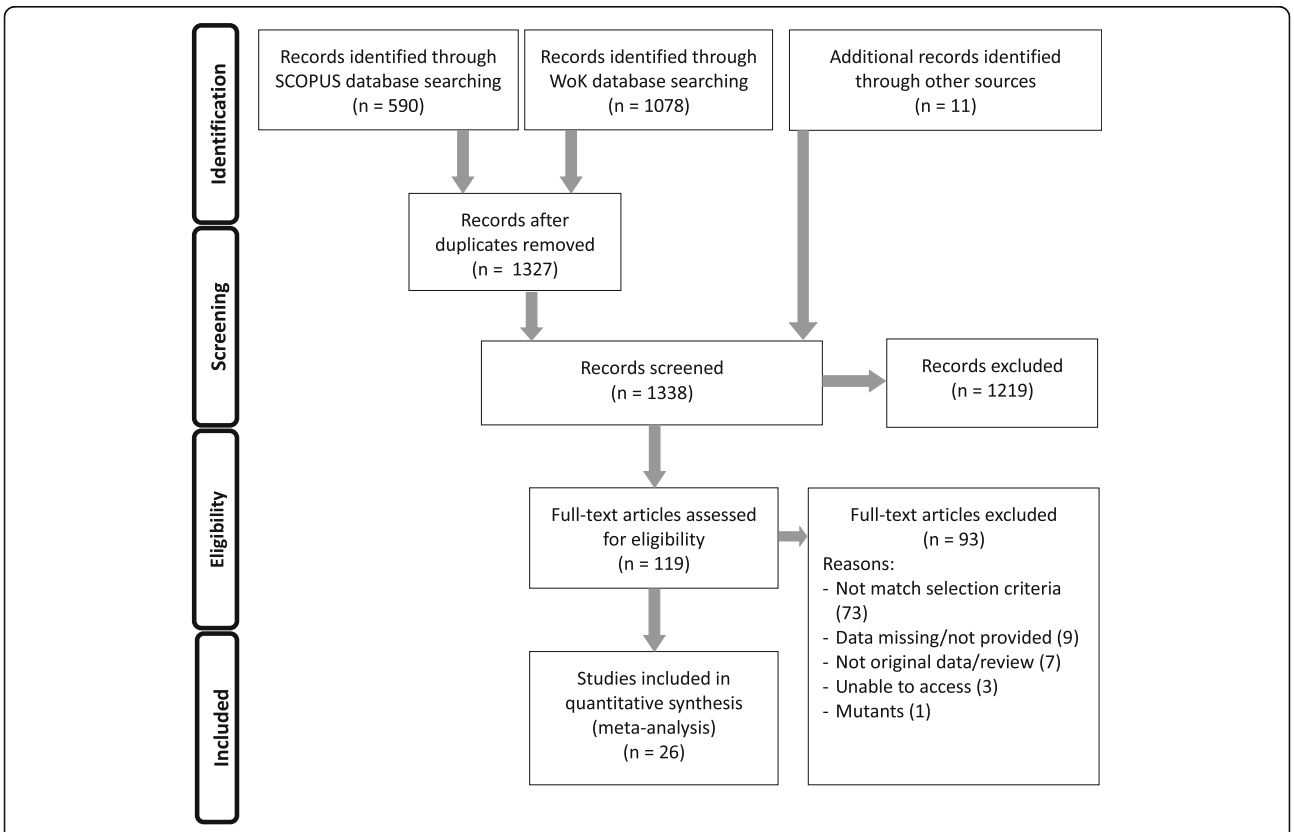

Fig. 1 PRISMA flow diagram of data collection. The number of papers identified initially through key word searching is shown in the identification boxes. The number of papers excluded is shown for each stage of screening. Reasons for exclusion are given for papers that made it to final eligibility screening

cost or high cost (i.e., on an ordinal scale, see Additional file 1: Table S1). This measure of cost was graded to take into account species and sex specific costs. For example, in male *D. melanogaster*, ejaculate production was classified as low cost, courtship for a single mating event as medium cost and lifetime courtship investment as high cost. Although subjective, we feel the use of three categories allowed reasonably accurate assignment of traits to a particular category and was necessary to assess how many studies allowed individuals to experience near total reproductive costs. Furthermore, when categorising the cost of trait, we took the study species into consideration, to account for differences in reproductive biology between different species and particularly differences between vertebrate and invertebrate reproductive biology. This also enables cross species comparison, despite the wide variety of reproductive traits being measured.

Statistical analysis

Analysis was carried out in R [31] using the packages *metaphor* [32] and *MCMCglmm* [33] implementing multi-level meta-analysis (MM) and phylogenetic multi-level meta-analytic models (PMM) [34, 35] (see Additional file 1: Dialog S1 for details). We first ran models without moderators to examine overall patterns and to compare phylogenetic and non-phylogenetic models. We then added single moderators to the models to examine their effects in isolation. Finally, we constructed a full model including all moderators of interest. In the results section, we present mean standardized difference between control and restricted groups, standard errors and 95 % credible intervals (CIs). When comparing phylogenetic models to non-phylogenetic models we present the Akaike information criterion (AIC), which is a model selection index, with the better model having a smaller AIC. Publication bias was examined through visual assessment of the data and through Eggers regression.

Results and discussion

Does DR reduce reproduction universally?

DR on average resulted in a significant reduction in reproduction (mixed-effect meta-analysis, MM: $\beta_{[\text{meta-analytic mean}]} = -0.841$, 95 % Confidence Intervals (CI) = [−1.374 to −0.308]). This effect remained robust even when the phylogenetic non-independence of the samples was accounted for (phylogenetic mixed effect meta-analysis, PMM: $\beta_{[\text{meta-analytic mean}]} = -0.841$, CI = [−1.374, −0.308], Additional file 1: Table S2). However, there was no evidence of a strong phylogenetic signal ($I^2_{[phylogeny]} <$ 0.001 %, Additional file 1: Table S3) in the effect of DR on reproduction, suggesting a consistent pattern across taxa. Although the model including phylogenetic signal was a better fit by AIC score (phylogenetic AIC = 577.33, non-phylogenetic = 579.86), the improvement was small and was not true for the model including all moderators (see below). To facilitate comparison we present models without phylogenetic signal included from here onwards; results are qualitatively the same for models including phylogenetic signal. Despite the small phylogenetic signal, we observed high heterogeneity amongst studies ($I^2_{[total]} = 98.65$ %, Additional file 1: Table S3), suggesting that the reduction in reproduction in response to DR was more apparent in certain studies. As stated above, such large heterogeneity (*sensu* [30]) calls for the use of moderators in our models to try to explain variation among studies.

Is there an effect of restriction severity?

As discussed above, an obvious pattern to explore is how reproduction responds to variation in the degree of restriction applied. In general, increasingly severe restrictions appear to increase the lifespan extension achieved by DR, up to the point of malnutrition. However, a linear change in reproduction is not predicted by existing evolutionary theories of DR. We tested these predictions by fitting both a linear and quadratic effect of the degree of restriction. We found a linear negative effect of the degree of restriction (BMM: $\beta_{[\text{Restriction}]} = -0.0158$, CI = [−0.0219, −0.0096], Fig. 2, Additional file 1: Table S4), but no significant quadratic effect (MM: $\beta^2_{[\text{Restriction}]} = -0.884$, CI = [−0.925, 2.694], Additional file 1: Table S4). This result is intriguing as it is counter to the predictions of both current evolutionary theories of DR [12, 29, 36]. One possible explanation for our inability to detect any non-linear pattern is a lack of data at particular restriction levels. Although many of the results analysed here were from studies with reasonably severe dietary restrictions (41 effect sizes, out of 205, with restriction levels greater than 75 % of *ad libitum*), there are very few data points with dietary restriction at *very* low or *very* high levels, particularly in model species (Fig. 2).

Is there a model species effect?

A recent meta-analysis demonstrated that DR is nearly twice as effective at extending life in model compared to non-model species [1]. We therefore tested whether such a model species effect was also apparent for reproduction. To allow direct comparison, we defined model species as the same five species used in the meta-analysis on lifespan [1] (*i.e.*, *R. norvegicus*, *M. musculus*, *D. melanogaster*, *C. elegans*, *S.cerevisiae*). Our results show that model species suffer a statistically significant reduction in reproduction (MM: $\beta_{[\text{model}]} = -2.42$, CI = [-3.41, -1.43], Fig. 3a, Additional file 1: Table S5), whereas the reduction in non-model species was lower and marginally non-significant (MM: $\beta_{[\text{non-model}]} = -0.445$, CI = [−0.926, 0.033], Fig. 3a, Additional file 1: Table S5). Comparing these effects, DR had a significantly stronger effect on

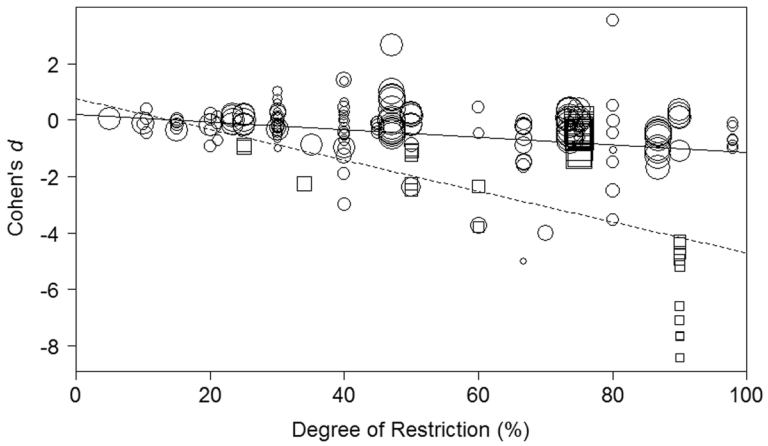

Fig. 2 The effect of degree of restriction on effect size in model and non-model species. Effect sizes are Cohen's *d*, the standardised mean difference in reproduction between the control and restricted groups (see Methods and Additional file 1: Dialog S1). Model species are represented by squares and the dashed line. Non-model species are represented by circles and solid line. Model species suffer a greater rate of decline in reproduction with increasing degree of restriction. Point sizes indicate the variance in the estimate of the effect size. Details of statistics are given in the main text

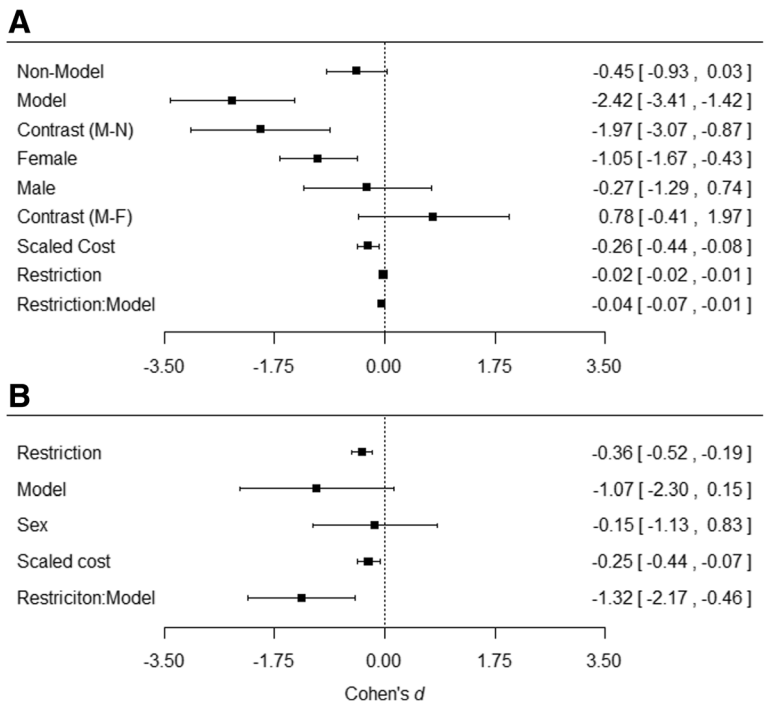

Fig. 3 Forest plots showing effect sizes (Cohen's *d*, standardised mean difference in reproduction between the control and restricted groups (see Methods and Additional file 1: Dialog S1)) of key moderators for the effect of dietary restriction (DR) on reproduction. Each point represents the Cohen's *d* value with the 95 % credible intervals (CIs). Panel **a** represents the outputs from univariate models, with each moderator fitted individually. Each moderator subgroup (e.g. model or non-model species) is represented by a single point. Contrasts represent the difference between effect sizes of the subgroups (e.g. the difference between model (M) and non-model (N) species). Restriction:Model, represents the interaction between degree of restriction (%) and model or non-model species. Panel **b** shows the output from our full model accounting for all moderators, with each point representing the effect size for that moderator

reproduction in model than non-model organisms (MM: $\beta_{[\text{non-model/model difference}]}$ = -1.97, CI = [−3.07, −0.87], Fig. 3a, Additional file 1: Table S5).

In an attempt to disentangle this effect further, we included the interaction between model organism and degree of restriction. This analysis revealed a statistically significant interaction (MM: $\beta_{[\text{restriction * model}]}$ = −0.0415, CI = [−0.0710, 0.0120], Figs. 2 and 3a, Additional file 1: Table S6); the rate of decline of reproduction with increasing DR was steeper in model than non-model species, suggesting that reproduction in model species is more responsive to resource availability than reproduction in non-model species. These results fit well with the findings of Nakagawa *et al.* [1] and with the disposable soma theory of the effect of DR on longevity, if this increased reduction in reproduction results in more resources being available for reallocation to somatic maintenance. However, the obvious question becomes why do model species have a greater reproductive response to increasing restriction than non-model species?

One possibility is that this is an unintentional effect of selection and subsequent adaptation to the laboratory environment [37]. For example, the laboratory environment is nutrient rich compared to the natural environment and selects for high fecundity but not longevity [38, 39]. Such an environment may inadvertently favour individuals that have greater plasticity in reproduction in response to nutrient availability. If such plasticity is maintained, either because it has no cost under laboratory conditions or because laboratory conditions vary enough to maintain plasticity, populations that have undergone generations of laboratory selection would be predicted to respond more plastically to food availability than populations that had not undergone such selection. On the other hand, natural environments may be predicted to be more variable than laboratory environments, particularly in food availability and this may be expected to select for increased plasticity in non-model species. Although a small number of studies compare the effectiveness of DR in extending lifespan in laboratory maintained populations versus wild or wild derived populations [37, 38, 40], results are inconsistent. It would therefore be interesting to increase the number of these studies and to use a range of food availabilities (rather than just two) to test whether laboratory populations are more plastic to food availability than wild derived populations. If so, inadvertent laboratory selection for high fecundity in a novel environment may have accounted for this plasticity.

Another possible explanation for the increased reproductive response to nutrient restriction in model species is that researchers can more effectively implement restriction in model species [1]. Model species have been studied in laboratory environments for many generations and thus diets are more likely to be optimised. In non-model species, where we know less about their nutritional requirements, "*ad libitum*" treatments may actually be fed to excess and foods are unlikely to be optimised. Thus when applying DR, the restricted group may be under much lower restriction levels than expected in non-model species. For example, a 75 % restriction may actually contain 90 % of the nutrients needed. Furthermore, the application of the geometric framework of nutrition to DR studies [41, 42], has provided a growing body of evidence that specific diet composition affect lifespan and reproduction and that this may be as, or even more, important than classical restriction (e.g. [2, 5, 27, 28]). Studies that use the same species may utilize diets with slightly different composition, which would undoubtedly affect results. It stands to reason, however, that model species which are frequently studied, will have better defined nutrient requirements and therefore that there may be less variation in diet composition and more consistent results. Obviously other explanations are possible, but our results and those of Nakagawa et al. [1] highlight the need for more research to investigate the cause of this model organism effect and how it may affect the generality of the conclusions drawn from investigations of DR.

Is there sexual dimorphism?

We next addressed whether there are sex differences in the reproductive response to DR, similar to those observed in the longevity response [1]. Our analysis revealed that females suffer a significant reduction in reproduction under DR (MM: $\beta_{[\text{female}]}$ = −1.05, CI = [−1.67, −0.43], Fig. 3a, Additional file 1: Table S7), but that this reduction is much smaller and statistically non-significant in males (MM: $\beta_{[\text{male}]}$ = −0.274, CI = −1.291, 0.742, Fig. 3a, Additional file 1: Table S7). However, when comparing the magnitude of the effect between the sexes, we found no statistically significant difference between males and females (MM: $\beta_{[\text{male/female difference}]}$ = 0.776, CI = [−0.414,1.967], Fig. 3a, Additional file 1: Table S7). The lack of statistical significance in comparison between the sexes is probably because of a lack of statistical power, with the sample size for males being particularly small, only 42 out of 205 effect sizes. These effect size estimates in males come from seven studies, covering five species, all of which were vertebrates (two bird species, one rodent, one primate and one fish species). The remaining studies were on females and there were no studies that allowed side-by-side comparisons of the effect of DR on males and females of the same species. Thus, studies that allow such direct comparison and generally more studies investigating DR in males would be desirable avenues of future research.

Does the cost of the reproductive trait measured matter?

It seems intuitive that traits which are more costly or encompass a greater proportion of total reproductive investment, such as lifetime egg production, will suffer a greater reduction under DR than low cost traits, such as producing a single ejaculate. We therefore included the estimated costliness of the reproductive trait as a moderator. High and moderate cost reproductive traits were statistically significantly reduced under DR (MM L: $\beta_{[high]} = -1.12$, CI = [−1.71, −0.54]; $\beta_{[moderate]} = -1.05$, CI = [−1.62, −0.48], Additional file 1: Figure S2 and Table S8). In contrast, low cost traits suffered a much smaller and statistically non-significant reduction under DR (MM: $\beta_{[low]} = -0.244$, CI = [-0.861, 0.374], Additional file 1: Figure S2 and Table S8). This result is unsurprising, but has implications for future DR studies. If, as the disposable soma theory of DR suggests, the effect on longevity is due to a decrease in reproduction, future experiments must allow both control and restricted individuals to experience and express high cost reproductive traits. Otherwise, if individuals are only exposed to a small proportion of the costs of reproduction, the differences between control and restricted individuals are expected to be smaller and more difficult to detect. This may be one explanation for the current sex difference in the effect of DR if females are exposed to more of the costs of reproduction than males (see also below).

This point becomes particularly relevant when examining the current data set in detail. As mentioned above, our search criteria resulted in only 42 effect sizes for males versus 163 for females. Of these 42, only 1 was classed as a high cost reproductive trait (a measure combining all reproductive behaviour into a single score of sexual activity), 18 were moderate cost and the remaining 23 were low cost. The distribution for female traits was: 77 high cost, 69 moderate costs and 17 low cost traits. Given the difference in distribution of the cost categories between males and females ($\chi^2_{2df} = 51.30$, $p < 0.001$), it is unclear if the above sex differences in the reproductive response to DR are real or simply reflect difference in the costs of traits that have tended to be measured in males and females. To test this we fitted a final, 'full' model, to assess the effect of the inclusion of all moderators considered on the estimated effects.

Putting it all together

When accounting for all of the individual moderators and the interaction between model species and the degree of restriction, the degree of restriction, the cost of the trait and the interaction were all statistically significant predictors of the reduction in reproduction under DR (MM: $\beta_{[Restriction]} = -0.357$, CI = [−0.520, −0.194]; $\beta_{[cost]} = -0.252$, CI = [−0.436, −0.067]; $\beta_{[restriction : model]} = -1.32$, CI = [−2.17, −0.47], Fig. 3b, Additional file 1: Table S9). This model had a conditional R^2 value of 78.8 % with random effects explaining 33.2 % and fixed effects explaining 45.6 % of the variation in effect size between studies [43]. When the interaction between model species and restriction was removed, restriction, model species and cost of trait remained as significant predictors (Additional file 1: Table S10).

As with the initial models, we also fitted models that accounted for the phylogenetic non-independence of species, with the non-phylogenetic model being the better fit (including interaction, phylogenetic AIC = 530.08, non-phylogenetic AIC = 528.08 (Additional file 1: Tables S9 and S11); excluding interaction, phylogenetic AIC = 539.22, non-phylogenetic AIC = 537.22 (Additional file 1: Tables S10 and S12)). This result suggests that the reduction in reproduction observed under DR is robust and phylogenetically conserved ($I^2_{[phylogeny]} < 0.001$ % Additional file 1: Table S13), but that the rate of reduction is greater in model species compared to non-model species. Furthermore, the reduction in reproduction was greater when examining more costly traits. Of particular interest when fitting the full model was the effect of including the cost of the trait on the sex difference in the effect of DR. When accounting for all other moderators, the difference between males and females was reduced (MM: $\beta_{[male/female\ difference]} = -0.151$, CI = [−1.132, 0.830] compared to MM: $\beta_{[male/female\ difference]} = 0.776$, CI = [−0.414, 1.967] in the model only containing sex, Fig. 3a and b). This result implies that the supposed sex differences in response to DR are being driven by experimental design, particularly the costs of reproduction experienced by the sexes.

Essential for all meta-analyses is the assessment of potential publication bias, as interpretation of results of meta-analyses assumes minimal publication bias in the literature [44]. Visual assessment of our data showed no obvious sign of publication bias (Additional file 1: Figure S3). Furthermore, statistical assessment revealed no significant publication bias in our data set once accounting for heterogeneity [35] (Eggers regression on the 'meta-analytic' residuals; $\beta_{[intercept]} = 0.0780$, S.E. = 0.0778, $p = 0.317$).

Conclusions

Our results represent the first formal meta-analysis of the effect of DR on reproduction, an important issue given some studies suggesting the effect of DR on longevity can be achieved independently of reproduction [17]. Above, we present three main findings that suggest explanations for outstanding issues in this field and avenues for future research. First, DR does lead to a reduction in reproduction but, in line with longevity [1], this effect is stronger in model species. We discuss a number of possible explanations for this phenomenon. However,

it is clear more studies are needed as any bias in patterns from model species as a result of laboratory adaptation have far reaching consequences for the role of DR studies in understanding and mitigating ageing and its application to humans [3]. Second, reproduction declines linearly with increasing DR, at odds with both current evolutionary theories of DR [12, 29, 38]. It is possible that our failure to detect a non-linear response of reproduction to DR was due to a lack of data at certain levels of restriction. More work across a broader range of restriction levels is needed to improve our power to detect non-linear effects and thus assess and compare alternative evolutionary hypotheses on DR effects [45, 46].

Finally, although our results support a sex difference in the response of reproduction to DR, they suggest this may be due to males and females being exposed to different levels of reproductive costs in the majority of experiments. An alternative explanation is that the longevity-reproduction trade-off can be uncoupled, with diets that maximize longevity not necessarily minimizing reproduction and that this effect can be sex specific [2, 28]. Definitive conclusions are difficult to draw because relatively few studies investigate the effect of DR on reproduction in males or allow direct comparison of males and females in the same study using a range of diets (but see [2, 28]). This is presumably because of the difficulty of designing meaningful measures of male reproductive investment that would encompass the majority of the costs. One potential solution is to measure many male reproductive traits and combine them into an overall score of reproductive investment [47]. Even if this is not possible, future DR studies must carefully consider the biology of the study organism and ensure both sexes are exposed to as close to the complete costs of reproduction as possible. For males this will usually include allowing costs such as those incurred while attracting females and direct competition with other males. By doing such experiments, we can start to assess whether sex differences in the response to DR, both in terms of reproduction and longevity, are a real and interesting sexual dimorphism, or an artefact of experimental design.

Additional file

Additional file 1: Further information is provided in Additional file 1.doc, which contains more detailed methods, supplementary figures and supplementary tables. (DOCX 100 kb)

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Availability of data and materials
The datasets and materials analysed during the current study are available in the Dryad repository, doi:10.5061/dryad.3fc02.

Authors' contributions
CAW and JPM conceived and designed the study, with input from SN on the design. Data collection was primarily performed by JPM with input from CAW. JPM and SN led the statistical analysis, but all authors contributed to the final analysis. Phylogenetic tree construction was carried out by ML. JPM wrote the initial draft of the manuscript and all authors contributed to editing the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
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Ethics approval and consent to participate
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References
1. Nakagawa S, Lagisz M, Hector KL, Spencer HG. Comparative and meta-analytic insights into life extension via dietary restriction. Aging Cell. 2012;11:401–9.
2. Jensen K, McClure C, Priest NK, Hunt J. Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in *Drosophila melanogaster*. Aging Cell. 2015;14:605–15.
3. Selman C. Dietary restriction and the pursuit of effective mimetics. Proc Nutr Soc. 2014;73:260–70. doi:10.1017/S0029665113003832.
4. Jiang JC, Jaruga E, Repnevskaya MV, Jazwinski SM. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. FASEB J. 2000;14:2135–7.
5. Lakowski B, Hekimi S. The genetics of caloric restriction in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A. 1998;95:13091–6.
6. Lee KP, Simpson SJ, Clissold FJ, Brooks R, Ballard JW, Taylor PW, Soran N, Raubenheimer D. Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. Proc Natl Acad Sci U S A. 2008;105:2498–503. doi:10.1073/pnas.0710787105.
7. Simons MJP, Koch W, Verhulst S. Dietary restriction of rodents decreases aging rate without affecting initial mortality rate – a meta-analysis. Aging Cell. 2013;12:410–4. doi:10.1111/acel.12061.
8. Colman RJ, Beasley TM, Kemnitz JW, Johnson SC, Weindruch R, Anderson RM. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Nat Commun. 2014;5:p3557.
9. Austad SN. Life extension by dietary restriction in the bowl and doily spider, *Frontinella pyramitela*. Exp Gerontol. 1989;24:83–92.
10. Terzibasi E, Lefrançois C, Domenici P, Hartmann N, Graf M, Cellerino A. Effects of dietary restriction on mortality and age-related phenotypes in the short-lived fish *Nothobranchius furzeri*. Aging Cell. 2009;8:88–99.
11. Kirkwood TBL. Evolution of ageing. Nature. 1977;270:301–4.

12. Shanley DP, Kirkwood TBL. Calorie restriction and aging: a life-history analysis. Evolution. 2000;54:740–50. doi:10.1111/j.0014-3820.2000.tb00076.x.
13. Williams GC. Natural selection, the costs of reproduction, and a refinement of Lack's principle. Am Nat. 1966;100:687–90. doi:10.2307/2459305.
14. Chippindale AK, Leroi AM, Kim SB, Rose MR. Phenotypic plasticity and selection in *Drosophila* life-history evolution. I. nutrition and the cost of reproduction. J Evol Biol. 1993;6:171–93.
15. Chapman T, Partridge L. Female fitness in *Drosophila melanogaster*: an interaction between the effect of nutrition and of encounter rate with males. Proc R Soc B. 1996;263:755–9.
16. Ball ZB, Barnes RH, Visscher MB. The effects of dietary caloric restriction on maturity and senescence, with particular reference to fertility and longevity. Am J Physiol. 1947;150:1188–92.
17. Mair W, Sgro CM, Johnson AP, Chapman T, Partridge L. Lifespan extension by dietary restriction in female *Drosophila melanogaster* is not caused by a reduction in vitellogenesis or ovarian activity. Exp Geront. 2004;39:1011–9. doi:10.1016/j.exger.2004.03.018.
18. Leroi MA. Molecular signals versus *Loi de Balancement*. Trends Ecol Evol. 2001;16:24–9.
19. Kaitala A. Dynamic life-history strategy of the waterstrider *Gerris thoracicus* as an adaptation to food and habitat variation. Oikos. 1987;48:125–31.
20. Boggs CL, Ross CL. The effect of adult food limitation on life history traits in *Speyeria Mormonia* (Lepidoptera: Nymphalidae). Ecology. 1993;74:433–41.
21. Inness CL, Metcalfe NB. The impact of dietary restriction, intermittent feeding and compensatory growth on reproductive investment and lifespan in a short-lived fish. Proc R Soc B. 2008;275:1703–8. doi:10.1098/rspb.2008.0357.
22. Burger JMS, Promislow DEL. Sex-specific effects of interventions that extend fly life span. Sci Aging Knowl Environ. 2004:pe30. doi:10.1126/sageke.2004.28.pe30.
23. Cooper TM, Mockett RJ, Sohal BH, Sohal RS, Orr WC. Effect of caloric restriction on life span of the housefly, *Musca domestica*. FASEB J. 2004;18:1591–3. doi:10.1096/fj.03-1464fje.
24. Magwere T, Chapman T, Partridge L. Sex differences in the effect of dietary restriction on life span and mortality rates in female and male *Drosophila Melanogaster*. J Gerontol A Biol Sci Med Sci. 2004;59:B3–9. doi:10.1093/gerona/59.1.B3.
25. Bonduriansky R, Maklakov A, Zajitschek F, Brooks R. Sexual selection, sexual conflict and the evolution of ageing and life span. Funct Ecol. 2008;22:443–53. doi:10.1111/j.1365-2435.2008.01417.x.
26. Vinogradov AE. Male reproductive strategy and decreased longevity. Acta Biotheor. 1998;46:157–60.
27. Carey JR, Harshman LG, Liedo P, Muller HG, Wang JL, Zhang Z. Longevity–fertility trade-offs in the tephritid fruit fly, *Anastrepha ludens*, across dietary-restriction gradients. Aging Cell. 2008;7:470–7.
28. Maklakov AA, Simpson SJ, Zajitschek F, Hall MD, Dessmann J, Clissold F, Raubenheimer D, Bonduriansky R, Brooks RC. Sex-specific fitness effects of nutrient intake on reproduction and lifespan. Curr Biol. 2008;18:1062–6. doi:10.1016/j.cub.2008.06.059.
29. Adler MI, Bonduriansky R. Why do the well-fed appear to die young? Bioessays. 2014;36:439–50. doi:10.1002/bies.201300165.
30. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ. 2003;327:557–60.
31. R Core Team. R: A language and environment for statistical Computing. Vienna, Austria. 2016. https://R-project.org/.
32. Viechtbauer W. Conducting meta-analyses in R with the metafor package. J Stat Softw. 2010;36:1–48.
33. Hadfield JD. MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. J Stat Softw. 2010;33:1–22.
34. Hadfield J, Nakagawa S. General quantitative genetic methods for comparative biology: phylogenies, taxonomies and multi-trait models for continuous and categorical characters. J Evol Biol. 2010;23:494–508.
35. Nakagawa S, Santos ES. Methodological issues and advances in biological meta-analysis. Evol Ecol. 2012;26:1253–74.
36. Mitteldorf J. Can experiments on caloric restriction be reconciled with the disposable soma theory for the evolution of senescence? Evolution. 2001;55:1902–5.
37. Harper JM, Leathers CW, Austad SN. Does caloric restriction extend life in wild mice. Aging Cell. 2006;5:441–9.
38. Miller RA, Harper JM, Dysko RC, Durkee SJ, Austad SN. Longer life spans and delayed maturation in wild-derived mice. Exp Biol Med. 2002;227:500–8.
39. Austad SN, Kristan DM. Are mice caloric restricted in nature. Aging Cell. 2003;2:201–7.
40. Metaxakis A, Partridge L. Dietary restriction extends lifespan in wild-derived populations of *Drosophila melanogaster*. PLoS One. 2013;8(9):e74681. doi:10.1371/journal.pone.0074681.
41. Simpson SJ, Raubenheimer D. Caoloric restriction and aging revisited: the need for a geometric analysis of the nutritional bases of aging. J Gerontol A Biol Sci Med Sci. 2007;62:707–13.
42. Simpson SJ, Raubenheimer D. Macronutrient balance and lifespan. Aging. 2009;1(10):875–80.
43. Nakagawa S, Schielzeth H. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods Ecol Evol. 2013;4:133–42.
44. Egger M, Smith GD, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. BMJ. 1997;315:629–34.
45. Tatar M. The plate half-full: status of research on the mechanisms of dietary restriction in *Drosophila melanogaster*. Exp Gerontol. 2011;46:363–8.
46. Flatt T. Plasticity of lifespan: a reaction norm perspective. Proc Nutr Soc. 2014;73:532–42.
47. Devigili A, Kelley JL, Pilastro A, Evans JP. Expression of pre- and postcopulatory traits under different dietary conditions in guppies. Behav Ecol. 2013;24:740–9.

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ORIGINAL RESEARCH

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Body macronutrient composition is predicted by lipid and not protein content of the diet

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Abstract

Diet is an important determinant of fitness-related traits including growth, reproduction, and survival. Recent work has suggested that variation in protein:lipid ratio and particularly the amount of protein in the diet is a key nutritional parameter. However, the traits that mediate the link between dietary macronutrient ratio and fitness-related traits are less well understood. An obvious candidate is body composition, given its well-known link to health. Here, we investigate the relationship between dietary and body macronutrient composition using a first-generation laboratory population of a freshwater fish, the three-spine stickleback (*Gasterosteus aculeatus*). Carbohydrate is relatively unimportant in the diet of predatory fish, facilitating the exploration of how dietary protein-to-lipid ratio affects their relative deposition in the body. We find a significant effect of lipid intake, rather than protein, on body protein:lipid ratio. Importantly, this was not a result of absorbing macronutrients in relation to their relative abundance in the diet, as the carcass protein:lipid ratios differed from those of the diets, with ratios usually lower in the body than in the diet. This indicates that individuals can moderate their utilization, or uptake, of ingested macronutrients to reach a target balance within the body. We found no effect of diet on swimming endurance, activity, or testes size. However, there was an effect of weight on testes size, with larger males having larger testes. Our results provide evidence for the adjustment of body protein:lipid ratio away from that of the diet. As dietary lipid intake was the key determinant of body composition, we suggest this occurs via metabolism of excess protein, which conflicts with the predictions of the protein leverage hypothesis. These results could imply that the conversion and excretion of protein is one of the causes of the survival costs associated with high-protein diets.

KEYWORDS
body composition, diet, dietary restriction, fat storage, nutrition

1 | INTRODUCTION

Variation in diet is well known to be a critical determinant of fitness-related traits such as growth, reproduction, and survival (Fontana & Partridge, 2015; Partridge, Gems, & Withers, 2005). In particular, dietary restriction (DR), a reduction in the intake of calories or particular macronutrients, has been shown to extend lifespan and protect against age-related diseases in the majority of species studied to date (see Speakman & Mitchell, 2011; Nakagawa, Lagisz, Hector, & Spencer, 2012; Selman, 2014 for recent reviews). It is widely accepted that this lifespan extension can be achieved through a reduction in calorie intake (McCay, Crowell, & Maynard, 1935; reviewed Speakman & Mitchell, 2011). However, recent research has rejuvenated the suggestion that variation in the ratio of specific macronutrients, and in particular a reduction in the protein content of the diet, is a key component of the relationship between diet and lifespan (Carey et al., 2008; Lee et al., 2008; Maklakov et al., 2008; Fanson, Weldon, Pérez-Staples, Simpson, & Taylor, 2009; Solon-Biet et al., 2014; Jensen, McClure, Priest, & Hunt, 2015; but see Speakman, Mitchell, & Mazidi, 2016; Simpson et al., 2017 for discussion). Despite this interest, the traits that link dietary macronutrient intake and lifespan are not currently known. An obvious starting point is the relationship between dietary macronutrient ratio and body composition, especially given the importance of body composition and particularly fat deposition, in determining health and lifespan (Barzilai, Banerjee, Hawkins, Chen, & Rossetti, 1998; Muzumdar et al., 2008). Here, using a freshwater fish as our model, we investigate the relationship between macronutrient ratio of the diet and body composition, as well as how macronutrient ratio impacts on physical performance and activity, two indicators of health and lifespan.

Calorie restriction is well known to affect body weight (McCay et al., 1935) but is also suggested to affect body composition, particularly adiposity (Colman, Roecker, Ramsey, & Kemnitz, 1998; Hempenstall, Picchio, Mitchell, Speakman, & Selman, 2010; Mitchell et al., 2015; Muzumdar et al., 2008; Picard & Guarente, 2005) and relative organ size (Mitchell et al., 2015; Selman et al., 2005). In fact, it has been suggested that a reduction in adiposity is the primary mechanism through which calorie restriction acts to extend health and lifespan (Barzilai et al., 1998; Muzumdar et al., 2008; Picard & Guarente, 2005). In mice, for example, adipose loss due to calorie restriction occurs in a graded manner, mirroring that of lifespan extension (Mitchell et al., 2015). However, contradictory evidence suggests that fat loss under calorie restriction provided no benefit or was detrimental to lifespan (Chiba et al., 2014; Liao et al., 2011; Park et al., 2017). Thus, although body composition appears to play a role in mediating the effect of calorie restriction on lifespan, the exact nature of this relationship is currently unclear.

Similar to calorie restriction, changes in dietary macronutrient composition result in changes to both body composition and lifespan. For example, it has been shown that mice fed high protein:carbohydrate ratio diets have reduced body fat (Huang et al., 2013; Solon-Biet et al., 2014; Sørensen, Mayntz, Raubenheimer, & Simpson, 2008), but surprisingly not the longest lifespan (Solon-Biet et al., 2014). However, a different study found little to no effect of changing dietary protein:carbohydrate ratio on body fat mass (Mitchell et al., 2015). In *Drosophila melanogaster*, body weight and lipid-free bodyweight increased with increasing protein:carbohydrate ratio of the diet, with carcass lipid content highest on a dietary protein:carbohydrate ratio of 1:2 (Lee, 2015). These flies had the second highest mean and maximum lifespans, with lifespan maximized on a 1:4 diet. However, additional studies in *D. melanogaster* found that with increasing protein intake, there was a decrease in body weight, due to a decline in body fat (Ponton et al., 2015; Skorupa, Dervisefendic, Zwiener, & Pletcher, 2008). Thus, as with calorie restriction, although dietary macronutrient ratio appears to influence body composition, the relationship between diet and body composition and lifespan appears complex.

Improving our understanding of how variation in dietary macronutrient ratio influences body composition may shed light on the causes of the lifespan cost of being fed imbalanced diets. An obvious candidate is that there are metabolic or storage costs of excess nutrients merely being absorbed in relation to their relative abundance in the diet. It is known that the body has a limited capacity for storing excess protein, with surplus nitrogen being excreted as urea (Delimaris, 2013; Heaney, 1998; Tarnopolsky et al., 1992). However, there is a positive relationship between fat intake and fat storage, with ingestion of high-fat diets resulting in increased fat storage and obesity and thus potentially the associated negative consequences for health and survival (reviewed Hariri & Thibault, 2010; but see Liao et al., 2011; Chiba et al., 2014; Park et al., 2017). The protein leverage hypothesis suggests that individuals eat primarily to obtain a target protein level, with carbohydrate and fat being overconsumed on low-protein diets in an attempt to reach this protein level (Huang et al., 2013; Simpson & Raubenheimer, 2005; Sørensen et al., 2008). Although this hypothesis focuses on protein intake, it can be predicted that this modification of food intake in relation to protein availability will also affect body composition (Simpson & Raubenheimer, 2005). For example, when eating to a target protein intake, nonprotein constituents are consumed in relation to their abundance in the diet. Therefore, across multiple diets with varying ratios of protein:nonprotein, we would expect the protein content of the body to remain stable, but the content of other components to vary in relation to their relative abundance. Studies from agriculture and aquaculture would seem to support this; when protein is limiting, individuals appear to prioritize protein ingestion and consequently overconsume lipid and carbohydrate, resulting in greater adiposity (Aletor, Hamid, Niess, & Pfeffer, 2000; Andrews & Ørskov, 1970; Donaldson, Combs, & Romoser, 1956; Ruohonen, Koskela, Vielma, & Kettunen, 2003; Ruohonen, Simpson, & Raubenheimer, 2007). If metabolic or storage costs of excess nutrients are driving the cost of imbalanced diets, we would expect that the protein:lipid ratio of the carcass would be similar to that of the diet and would have the same rank order of protein:lipid ratios as the diets.

An alternative explanation for the survival cost of imbalanced diets is that animals have the potential to selectively absorb and or excrete particular nutrients and that the cost of an imbalanced diet is due to the costs of these selective processes (Fanson, Fanson, & Taylor, 2012). Under this scenario, body and diet macronutrient compositions

would not be expected to match, but body compositions would be expected to be more similar than diet compositions, as individuals selectively absorb or excrete particular nutrients in attempt to reach a target protein:lipid ratio within the body. If individuals are targeting a specific carcass protein:lipid ratio, then the protein content of the carcass would differ across diets. Furthermore, we would expect to see clustering and a reduction in variability in carcass protein:lipid ratio, as individuals would be trying to achieve a particular protein content in relation to their lipid content.

In addition to body composition, physical activity and performance (e.g., endurance) are commonly linked with health and lifespan and are affected by diet. It has been suggested that an increase in activity in response to short-term food shortage would improve an individual's ability to find new food sources, thus explaining the commonly observed biphasic pattern of activity (reviewed Speakman & Mitchell, 2011). However, recent evidence suggests that the effect of calorie restriction differs between different components of activity (Mitchell et al., 2016). Currently, there is little to no exploration of how shortage of a specific macronutrients, rather than overall calorie deficit, affects activity and endurance.

Finally, the effect of diet appears to be sexually dimorphic, with lifespan extension under DR greater in females than males (Nakagawa et al., 2012 but see Speakman et al., 2016). It is thought that this sex difference is a result of a differences between males and females in their investment in reproduction (Shanley & Kirkwood, 2000; but see Moatt, Nakagawa, Lagisz, & Walling, 2016), but work exploring the effect of DR on reproduction in males is often lacking (Moatt et al., 2016). One measure of reproductive investment in males is testes mass, but this is often difficult to study as it would require sacrificing males in studies where lifespan is the key trait of interest. In mice, it has been shown that testes mass is only reduced at high restriction levels, suggesting testes are protected against the effect of DR (Mitchell et al., 2015). The same study reported a marginal effect of protein restriction on testes mass (Mitchell et al., 2015), with a further study reporting increased testes mass on high protein:carbohydrate ratios (Solon-Biet et al., 2015). However, very few other studies look at the effect of dietary macronutrients on testes mass.

Here, we used three-spined sticklebacks (*Gasterosteus aculeatus*) reared on diets that varied in macronutrient ratio to investigate the following questions: (1) what is the effect of macronutrient intake on growth and body composition and is this driven by variation in protein content of the diet; (2) how does macronutrient manipulation affect activity and swimming endurance; (3) are there sex differences in the effect of macronutrient manipulation; and (4) what is the effect of macronutrient manipulation on testes size? We predicted that growth would be highest on the diet with the best balance, containing high levels of both protein and lipid. In line with the protein leverage hypothesis, we expect the rank order of carcass protein:fat ratios will match that of the diet. Furthermore, we expect dietary protein content to predict carcass fat content but not carcass protein content, with little difference in carcass protein content across treatments. Thus, the protein content of the diet will predict carcass body composition. Furthermore, we expected carcass fat content to be higher with high lipid intake and low protein intake. For endurance and activity, we predicted that endurance would be greater on high-protein diets, as protein is important for muscle development while activity would be higher on low-protein diets to allow protein-restricted individuals to locate better food sources. Finally, we predicted that testes size would be larger on high-protein diets.

2 | METHODS

2.1 | Husbandry

Experimental individuals were first-generation offspring of wild-caught three-spine sticklebacks. Parents were collected in the spring of 2014 form Inverleith Pond, Edinburgh (55.96N 3.22W). Using standard IVF techniques for this species (Barber & Arnott, 2000), 23 clutches were produced, each with a unique sire and dam. Offspring were fed live *Artemia* until one month of age, after which they were provided live *Artemia* and fry powder (ZM Sytems, ZM-100 Fry Food: protein 55.0%, oil 13.0% and ash 12.0%) until 3 months of age. From three to four months (the start of dietary manipulations), fish were fed standard-grade fish pellet (ZM Systems, medium granular: protein 52.0%, oil 12.0% and ash 10.3%) to condition them to surface feeding on fish pellet. At 4 months of age, fish were molecularly sexed from fin clips and weighed. Fish were then randomly assigned to one of five diet treatments, such that an equal number of males and females were assigned to each diet. A total of 150 fish were used, giving 15 fish per sex per diet.

Fish were housed in plastic tanks (30 × 20 × 20 cm), provisioned with an individual air filter and two artificial weeds. Each tank contained three unrelated individuals of the same sex. Individuals were of a different size to enable individual identification of the fish without physically marking them (Lee, Monaghan, & Metcalfe, 2013). Clutches were evenly split between the tanks to control for both tank and family effects. Light and temperature regimes were matched to natural levels in Edinburgh at that time of year.

2.2 | Diet treatments

Unlike for mice and flies, where most work on macronutrient ratio has been carried out, it has been shown that carbohydrate is not a key macronutrient for predatory fish, with much more importance placed on lipid (Ruohonen et al., 2003). Therefore, we created five diets differing in the ratio of protein:lipid (Table 1). We suggest that in these diets, protein and lipid are not strongly negatively correlated (see Fig. S1), and thus allow us to separate the effect of diet into the independent effects of protein and lipid. To achieve this lack of correlation, we used inert carbohydrate filler, which has been shown to be indigestible in teleosts (Guillaume, 2001; Kim & Kaushik, 1992). Thus, although the diets differ in carbohydrate content (Table 1), this was indigestible to the fish. To test for a correlation between protein and lipid, we use their relative abundance (%) in the raw diet (g). However if you

TABLE 1 Table of the nutrient content of the five diets used in this experiment. Calories represent the usable energy in the diet, that is, the energy from protein and lipid only, excluding the indigestible carbohydrate. Macronutrient values are percentages of raw materials (g) in the diet (see Table S1 for details of energetic contributions of each nutrient)

Protein (%)	Lipid (%)	Carbohydrate (%)	Ratio P:L	Calories (MJ/kg)
67.5	6.6	15.8	10.2:1	13.8
33.2	3.9	53.1	8.5:1	7.1
59.3	13.0	16.1	4.6:1	14.8
51.6	20.5	17.8	2.5:1	16.3
31.2	19.2	39.7	1.6:1	12.4

consider the contribution of protein and lipid to usable energy, there is a strong negative correlation (see Table S1). We suggest our approach of considering relative abundance is more appropriate, as we quantify amounts of protein and lipid in body, not energy, and fat will be prioritized as an energy source with protein as a source of structural components, for example, amino acids for growth (see theory of protein sparing: De Silva, Gunasekera, & Shim, 1991; Vergara, Robainà, Izquierdo, & De La Higuera, 1996; Helland & Grisdale-Helland, 1998 and below). Diets were in pellet form made of different combinations of fish meal and fish oil (Table S2). Diets were manufactured at the Aquaculture and Fish Nutrition Centre (University of Plymouth, Plymouth, U.K.).

In the majority of studies where macronutrients are manipulated, diets are provided ad libitum with food available at all times. However, as food degrades rapidly in water, this feeding regime is not suitable for aquatic organisms. We therefore adapted a previous feeding regime that has been successful in fish (Terzibasi et al., 2009). Here, fish are fed to satiation twice per day, in the morning and in the evening. The amount of food provided for each diet was reassessed monthly, by feeding fish incrementally until satiated. This amount of food was then provided morning and evening for a month until the next reassessment was made. All tanks of the same diet were fed the maximum amount of pellet consumed by any tank on that diet. As a result, the majority of tanks were fed to excess with not all of the food ration being eaten; thus, we cannot quantify how much of the ration was consumed. Therefore, we do not present intake data on an individual or a tank level (e.g., Solon-Biet et al., 2014). Fish were maintained on diet treatments throughout the course of the experiment (106 days).

2.3 | Growth and condition

From the start of diet treatments until the end of the study, fish were weighed and length was measured approximately once a month. However, as growth was roughly linear (see Fig. S2), we only analyzed initial weight, to check for any differences between treatments before the start of the experiment, and final weight, to assess differences in growth between diet treatment. Furthermore, a common measure of assessing overall health of a fish is condition index. Here, we calculated condition using residuals from an analysis of the length–weight relationship (see Bentley & Schindler, 2013):

$$\text{Condition Index} = \log(\text{Weight}) - \log(a) - b\log(\text{Length})$$

with the slope (b) and intercept (a) taken from a model of the log of weight against the log of length for all fish measured in this study (Bentley & Schindler, 2013). A negative value indicates a fish in a poorer than average condition, and a positive value suggests a better than average condition.

2.4 | Swimming endurance

On one occasion between days 79 and 100, each fish was assessed for their swimming endurance ability. We used the same protocol as described in Alvarez and Metcalfe (2005). Briefly, fish were placed in a swim chamber (length 25 cm, internal diameter 6 cm) submerged in a glass-sided tank (59 × 29 × 28 cm) filled to a depth of 22 cm with room temperature water. Fish were exposed to two currents, generated within the swim chamber, initially a slow current (4 cm/s) for 5 min, to condition individuals to the swim chamber, after which the speed was increased to 20 cm/s and a timer started. At the first cessation of swimming, fish were prompted to return to swimming by a small tap on the chamber. If this failed to elicit swimming, or at the second refusal to swim, the current and timer were stopped. Where individuals continued to swim, the trial was allowed to run for a maximum of 30 min (5 min acclimatization and 25 min at 20 cm/s). Immediately following the trail, the fish was removed to a recovery tank and a 50% water change performed before another trial was initiated. Temperature was recorded every two hours and then converted into a daily average. Swimming endurance was taken as the time an individual was able to remain swimming while exposed to the high-speed current, and any fish that swam for the full trial was given a score of 25 min (23 of 118 tested). Swimming endurance tests were performed with the observer blind to dietary treatment.

2.5 | Activity

To assess the effect of diet on levels of activity, activity trials were conducted between days 79 and 100. Activity trials were carried out in a glass-sided tank (45 × 25 × 25 cm), containing water to a depth of 8 cm following a similar protocol to Boulton, Grimmer, Rosenthal, Walling, and Wilson (2014). The tank was placed on a light box, surrounded by white walls to prevent disturbance and a video camera mounted above the tank. Each fish was placed in the center of the tank and given a 60-s acclimatization period, followed by eight-minute monitoring. Fish activity was tracked using Viewer^3 tracking software (http://www.biobserve.com/behavioralresearch/products/viewer/). Activity was measured as the total time spent moving during the eight-minute assessment window. Following the assessment period, the fish was removed and a 100% water change was performed prior to the next trial, thereby ensuring there were no chemical cues remaining in the water which could affect the next trial.

2.6 | Testes mass

At the end of the experiment (24/02/2015), all males were sacrificed through overdose of tricaine mesylate (MS222) and physical destruction of the brain. They were dried, by blotting with paper towel, and then both testes were removed and transferred to a preweighed Eppendorf. Owing to the delicate nature of the testes, they were not dried prior to weighing. The Eppendorf was then reweighed on a fine balance (±0.001 g), and testes mass was taken as the difference between the two weights (g). Testes measurements were carried out with the observer blind to dietary treatment.

2.7 | Body composition

On the 25/02/2015, all female fish were also sacrificed through overdose of MS222 and physical destruction of the brain. Carcasses of both sexes were frozen at −20°C until carcass composition analysis was carried out. Wet and dry mass of carcasses were quantified. Soxhlet extraction was used to quantify the fat mass and fat-free mass (protein mass), and the remaining carcass was then ashed to determine the bone and mineral content of the samples. We therefore quantified body composition as protein content (g), lipid content (g), ash content (g), and the ratio of protein:lipid in the carcass. Analyzing three measures of body composition (ratio of protein:lipid, protein content, and lipid content) allows us to test whether changes in the ratio of macronutrients in the body are driven by variation in protein content, lipid content, or both. Body composition was analyzed blind of the dietary manipulations.

2.8 | Statistical analysis

All analyses were carried out in R (v3.3.1; R core team, 2016) using the packages *Lme4* (Bates, Mächler, Bolker, & Walker, 2015) and *MCMCglmm* (Hadfield, 2010). Tank and family of origin were included as random effects in all models. The ratio of protein:lipid in the carcass was analyzed via linear mixed effects (LME) models with diet and sex included as categorical fixed effects. Carcass protein, carcass lipid, and carcass ash contents were analyzed via LME models, with diet and sex included as categorical fixed effects and carcass dry weight included as a continuous covariate to account for differences in size. Protein and lipid content of the diets were not strongly negatively correlated (see Fig. S1); therefore, we fitted models to try to separate the effects of dietary protein and lipid. These models included the same fixed and random effects as above, but with dietary protein and lipid included as continuous covariates in place of diet. Testes mass was analyzed via LME with diet as a categorical fixed effect and wet weight included as continuous variable. LME models for wet and dry weight contained diet and sex as categorical fixed effects. To assess the effect of diet on activity, we analyzed total time moving using LME models with diet and sex as factors and wet weight as a covariate. Swimming endurance was analyzed via a Markov chain Monte Carlo generalized linear mixed model (MCMCglmm) using a censored exponential distribution, because this data were exponentially distributed, with a number of fish swimming for the full 25 minutes. To minimize autocorrelation of the model, it was run for 1,300,000 iterations and a burnin of 300,000 with 1,000 samples stored. Diet, sex, wet weight and water temperature were included as fixed effects, and tank was included as a random effect.

3 | RESULTS

3.1 | Growth

There were no significant differences in initial weight or length between the treatments (LME; weight: $\chi^2 = 2.11$; $p = .716$; Fig. S2; length: $\chi^2 = 1.33$; $p = .857$). However, there was a marginally nonsignificant difference between the sexes in initial weight (LME; $\chi^2 = 3.38$; $p = .066$) and a significant effect of sex on initial length (LME; $\chi^2 = 4.75$; $p = .029$), with females being slightly larger than males (mean weight (g) ± *SE*: females 0.43 ± 0.02; males 0.38 ± 0.02; mean length (mm) ± *SE*: females 34.20 ± 0.64; males 32.58 ± 0.58). The marginally nonsignificant difference in initial weight between the sexes disappeared by the final weighing (LME; $\chi^2 = 0.98$; $p = .323$) but remained significant for length at final measuring (LME; $\chi^2 = 4.21$; $p = .040$; mean length (mm) ± *SE*: females 44.60 ± 0.64; males 42.96 ± 0.79). There was a significant effect of diet on final weight (LME; $\chi^2 = 18.44$; $p = .001$; Figure 1) and final length (LME; $\chi^2 = 13.43$; $p = .009$). Post hoc analysis revealed fish on the *2.5:1* diet were significantly heavier than those on all other diets (Table S3), but longer only than fish on the *8.1:1* diet (Table S4, Fig. S3). However, there was no difference in weight or length for all other diet comparisons (Figure 1, post hoc analysis Tables S3 and S4, Fig. S3). Diet also had a significant effect on dry weight (LME; $\chi^2 = 28.26$; $p < .001$), with post hoc analysis again revealing this difference was driven by fish on the *2.5:1* diet being significantly heavier than fish on all other diets (post hoc analysis Table S5). As with wet weight, there was no effect of sex on dry weight of the carcass at the end of the experiment (LME; $\chi^2 = 28.26$; $p = .197$).

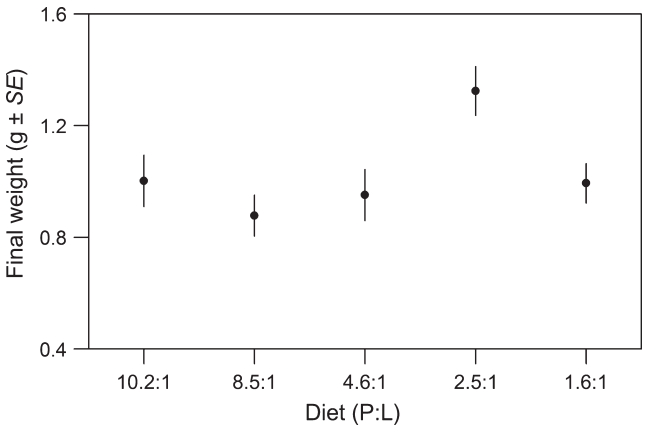

FIGURE 1 Mean final weight (g ± *SE*) in relation to diet (protein:lipid). There was an effect of diet on final weight ($p = .001$), with individuals on diet 2.5:1 significantly heavier than individuals reared on all other diets (all $p < .040$). There were no differences between the weight of individuals reared on the remaining four diets (all $p > .6$)

As with final weight, there was a significant effect of diet on condition index. However, the pattern of differences between treatments for condition index was not the same as that of weight and length. Fish on the *4.6:1* diet were in significantly poorer body condition than fish on the *8.5:1* and *2.5:1* diets, and a poorer but marginally nonsignificant condition to fish on the *1.6:1* diet (post hoc comparisons Table S6; Figs. S4 and S5). There were no significant differences in condition for all remaining comparisons (Table S6). As with final weight, there was no effect of sex on condition index ($p = .260$).

3.2 | Body composition

Analysis of the ratio of protein:lipid in the carcass revealed a significant effect of diet (LME; $\chi^2 = 38.60$; $p < .001$; Figure 2; post hoc Table S7). Interestingly, the protein:lipid ratio in the carcass did not match that of the diet, nor show the same rank order. The ratio of protein:lipid was lower in the fish than in the diet that they had consumed, with the biggest difference in fish from the highest protein:lipid diet (Figure 2a). To test this, we analyzed the difference between the protein:lipid ratio of the diet and that of the carcass of fish fed on that diet. There was indeed a significant effect of diet. Fish fed on high protein:lipid ratio diets had more of a difference between their body composition and the composition of the diet than fish fed on lower protein:lipid ratio diets (LME; $\chi^2 = 118.59$; $p < .001$; post hoc analysis Table S8; Fig. S6).

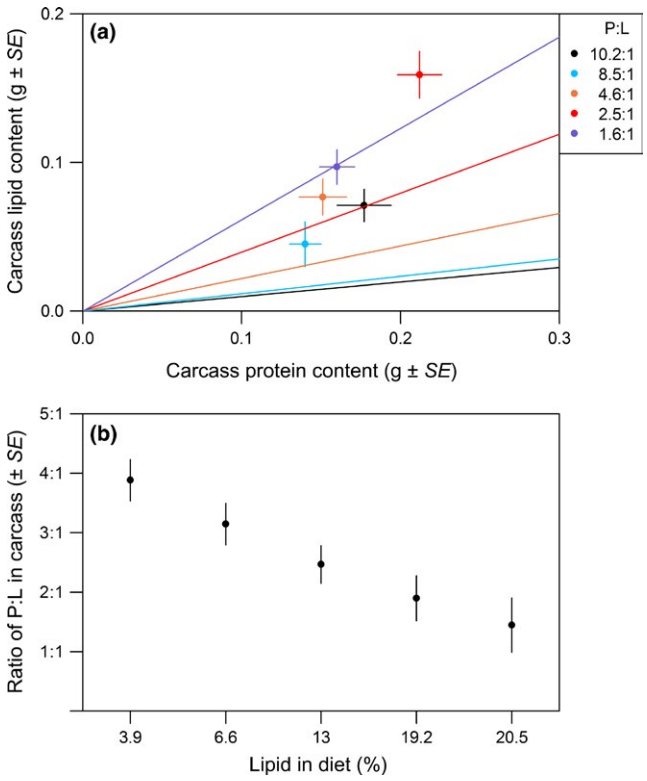

FIGURE 2 (a) Mean (±*SE*) carcass lipid content (g) against mean (±*SE*) carcass protein content (g). Rails represent the protein:lipid ratios in the five diets. Colors correspond to the five diets (see key). There was a significant effect of diet on the degree of difference between carcass and dietary protein:lipid ratio ($p < .001$). (b) Mean (±*SE*) carcass protein:lipid ratio in relation to dietary lipid (%). Ratio in carcass is carcass protein (g)/carcass lipid (g). Ratio of protein to lipid in the carcass decreased linearly with increasing dietary lipid intake ($p < .001$) but is not significantly affected by protein intake ($p = .180$)

Investigating the effect of the protein and lipid content of the diet separately revealed that the carcass protein:lipid ratio was significantly linearly influenced by the percentage of lipid in the diet (LME; $\chi^2 = 37.16$; $p < .001$), but not the percentage of protein (LME; $\chi^2 = 1.79$; $p = .180$; Fig. S7), with the protein:lipid ratio of the carcass decreasing with increasing lipid content of the diet (Figure 2b). Carcass protein:lipid ratio also differed between the sexes (LME; $\chi^2 = 4.54$; $p = .033$), with males having a lower ratio than females (mean ratio of protein:lipid ± *SE*: males 2.3:1 ± 0.1, females 2.9:1 ± 0.2).

Similar patterns were observed when independently analyzing the protein and lipid content of the carcass rather than their ratio. Diet had a significant effect on both protein (LME; $\chi^2 = 53.06$; $p < .001$; post hoc analysis Table S9) and lipid content (LME; $\chi^2 = 42.59$; $p < .001$; post hoc analysis Table S10) of the carcass when controlling for variation in dry weight (LME: Protein: $\chi^2 = 381.52$; $p < .001$. Lipid: $\chi^2 = 261.91$; $p < .001$), with protein content of the carcass increasing and lipid content decreasing as the dietary ratio of protein:lipid increased (Fig. S8). However, as with carcass protein:lipid ratio, this was driven by a linear effect of dietary lipid content, rather than an effect of dietary protein content: There was a negative linear effect of dietary lipid on carcass protein and a positive effect on carcass lipid (LME; Carcass protein $\chi^2 = 38.23$; $p < .001$; Carcass lipid $\chi^2 = 37.50$; $p < .001$; respectively; Fig. S8), but no effect of dietary protein (LME: Carcass protein $\chi^2 = 0.28$; $p = .600$; Carcass lipid $\chi^2 = 0.17$; $p = .677$; Fig. S8). Finally, there was a significant effect of sex on carcass lipid content (LME; $\chi^2 = 7.76$; $p = .005$), with males having greater lipid content of the carcass (mean lipid content (%) ± *SE*: males 28.09 ± 1.10, females 24.72 ± 1.20). However, the effect of sex was marginally nonsignificant for protein content (LME; $\chi^2 = 3.68$; $p = .055$), suggesting that ash content must differ. We therefore analyzed ash content, which is a measure of carcass bone and mineral content. There was a significant effect of sex on ash content (LME; $\chi^2 = 5.00$; $p = .025$), with females having greater ash than males (mean ash content (%) ± *SE*: males 15.09 ± 0.63, females 16.91 ± 0.63).

3.3 | Testes mass

There was a positive linear effect of final weight on testes mass (LME; $\chi^2 = 13.17$; $p < .001$; estimate ± *SE* (g): 0.00401 ± 0.00111). Accounting for final weight, there was no effect of diet on testes mass (LME; $\chi^2 = 3.96$; $p = .412$). However, despite the effect of diet on final weight, there was no evidence of an indirect effect of diet on testes mass, as diet was still nonsignificant when final weight was excluded from the model (LME; diet: $\chi^2 = 0.864$; $p = .930$).

3.4 | Swimming endurance and activity

The censored exponential model revealed no significant effect of diet, sex, weight, or water temperature on swimming endurance (MCMCglmm; all $p > .08$; Table S11). To assess activity, we analyzed

total time spent moving during the eight-minute assessment window. This revealed no significant effect of diet, sex, or weight on activity level (LME; Diet: $\chi^2 = 3.07$; $p = .547$; Sex: $\chi^2 = 0.691$; $p = .406$; Weight: $\chi^2 = 0.844$; $p = .358$; Table S12).

4 | DISCUSSION

Diet is known to be an important determinant of key fitness traits (Fontana & Partridge, 2015; Partridge et al., 2005). However, what mediates this effect is much less well understood. Our study explores the relationship between dietary macronutrient ratio and the macronutrient composition of the body, a key determinant of fitness traits such as health and lifespan. In particular, we explore the direct effect of dietary protein and lipid intake on protein and lipid content in the body. Interestingly, our findings suggest that individuals are able to alter their utilization or uptake of ingested macronutrients, with the ratio of protein:lipid in the carcass being vastly different from that of the diet. Furthermore, we found no effect of dietary protein intake on body composition, rather carcass protein and lipid content was predicted only by dietary lipid intake. Although the protein leverage hypothesis focuses on protein intake, these results conflict with our predicted outcomes of this for body composition (Simpson & Raubenheimer, 2005). Under the protein leverage hypothesis, we expected the rank order of diet protein:lipid ratios to be maintained in the ratio of protein:lipid in the carcass. Furthermore, we expected that the protein content of the diet would predict carcass body composition and the relative protein content of the body would be relatively stable. However here, there was no effect of protein intake on body composition, the rank order of protein:lipid ratios was not maintained from the diet to the carcass, and the protein content of the body varied across diets.

These findings have striking implications for studies exploring the relationship between diet and health or organismal fitness. It has been suggested that being consigned to a specific diet, but fed ad lib, allows individuals to increase or decrease their intake of that diet, but prevents them from altering the ratio of macronutrients they ingest (Simpson & Raubenheimer, 1995, 2007; Simpson, Sibly, Lee, Behmer, & Raubenheimer, 2004). However, our results show individuals clearly alter their utilization or uptake of the ingested macronutrients, resulting in vastly different macronutrient ratios in the carcass compared to the body. Furthermore, the range of protein:lipid ratios was 1.4:1 to 3.9:1 in the carcasses, but was 1.6:1 to 10.2:1 in the diets. This suggests a pattern of modification toward a lower and less variable carcass protein:lipid ratio. Previous work has suggested that lifespan is maximized on low protein:nonprotein intakes, with high-protein diets negatively affecting lifespan (Carey et al., 2008; Fanson et al., 2009; Jensen et al., 2015; Lee et al., 2008; Maklakov et al., 2008), which could imply that individuals are targeting lower protein:nonprotein ratios in an attempt to increase fitness.

Previous research suggests a survival cost to being maintained on an imbalanced diet. Two obvious alternative explanations for this are the cost of storage of excess nutrients or the cost of their selective absorption or excretion. Our results provide some support for the latter. Individuals fed diets of vastly different macronutrient ratios appeared to converge on more similar body compositions. This suggests that nutrients are not simply stored in proportion to their availability in the diet and thus that survival costs of imbalanced diets are likely associated with selective absorption or excretion of particular nutrients. Given that here, dietary lipid content, not protein, is driving body composition and the positive association between dietary lipid intake and adiposity (Hariri & Thibault, 2010), we suggest that this modification is achieved via metabolism of excess protein. The body has a limited capacity for storing excess protein, which must be converted into urea and excreted (Delimaris, 2013; Heaney, 1998; Tarnopolsky et al., 1992) which may represent one potential cost of a high-protein diet (Fanson et al., 2012).

Our results also provide mixed support for the well-known theory of protein sparing in fish, where individuals prioritize lipid use for energy expenditure and use protein for growth and muscle development (De Silva et al., 1991; Helland & Grisdale-Helland, 1998; Vergara et al., 1996). The lack of an effect of protein content of the diet on protein content of the carcass suggests individual fish were able to maintain the protein content of their carcass on protein intakes as low as 31.2% and conforms to the theory of protein sparing. However, the negative linear effect of lipid intake on carcass protein content is counter to predictions from protein sparing.

There was little effect of diet on growth, despite diets of differing energy levels being well known to affect size (e.g., Colman et al., 1998; McCay et al., 1935). However, in our study, food was provided ad libitum, meaning that despite the diets differing in energy content (Table 1), fish on lower energy diets could increase their intake and avoid caloric restriction. Only fish on the *2.5:1* diet were different in size, being significantly larger than all other fish in all other diets. Interestingly, the protein:lipid ratio in this diet is closest to the ratio that maximizes growth in European Whitefish, *Coregonus lavaretus* (Ruohonen et al., 2003). Ruohonen et al. (2003) suggested that growth was maximized on a 2.25:1 protein:lipid ratio as this feed had a high energy value. However, this explanation is unlikely here, as food was provided ad lib (see above), and there were no differences in growth between other diets differing greatly in energy content (e.g., 7.1 MJ/kg to 14.8 MJ/kg). We suggest that the *2.5:1* diet resulted in the greatest growth because it had the highest energy content in combination with a balance of protein and lipid and that high levels of no single dietary component can generate high levels of growth.

Our results also provide evidence of sexual dimorphism in body composition, with males being significantly shorter and having greater fat deposits, and females being longer and having higher bone and mineral deposits (indicated by the higher ash content). These findings fit with a previous study (Kitano, Mori, & Peichel, 2007), where female *G. aculeatus* were also found to be longer than males. We suggest that this is likely a result of the different reproductive behaviors exhibited by the sexes. When reproducing, male three-spine sticklebacks defend territories, construct nests, court females, and fan eggs, which likely impacts on their ability to forage (Wootton, 1984). Therefore, males potentially invest in fat

deposition, rather than growth in length, to provide greater energy reserves prior to the breeding season. This would explain the higher fat content of males here, as our fish were culled immediately prior to the breeding season.

We found no effect of diet on swimming endurance or activity, despite calorie restriction being known to affect activity and endurance (reviewed Speakman & Mitchell, 2011). However, individuals in the current study were fed ad libitum and could therefore obtain sufficient energy to maintain activity levels. Additionally, as discussed above, fish appeared able to selectively utilize their ingested macronutrients and therefore may not have been under major macronutrient imbalance; thus, there was no stimulation to increase activity levels. Alternatively, these findings could suggest that the effects of calorie restriction on performance are not reproducible through macronutrient manipulations. It is also possible that any differences in activity and endurance were too subtle to be detected in the current study.

Finally, we found no direct or indirect effect of diet on testes mass. This could reinforce the suggestion that the testes are protected from the effect of diet (Mitchell et al., 2015). Alternatively, it could suggest that testes size in the three-spine stickleback is a low-cost reproductive trait, and thus that the effect of diet is correspondingly small and therefore difficult to detect (Moatt et al., 2016).

In conclusion, we show that body macronutrient composition differs from that of the diet and that this pattern of variation suggests individuals are attempting to achieve a particular protein:lipid ratio in the body rather than prioritising a single macronutrient. We suggest individuals are achieving a balance of protein and lipid in the body by excreting excess protein. In contrast to a number of recent studies and the protein leverage hypothesis (Huang et al., 2013; Lee, 2015; Ponton et al., 2015; Skorupa et al., 2008; Solon-Biet et al., 2015; Sørensen et al., 2008), our results suggest lipid intake is the key determinant of body composition, rather than protein. Together, these data suggest that the key macronutrient for determining body composition may differ between species, which, if this extends to lifespan, has striking implications for studies of DR, where effects have been suggested to be evolutionarily conserved (e.g., see Moatt et al., 2016; Nakagawa et al., 2012). The results presented here seem to conflict with predicted outcomes of the protein leverage hypothesis, but we do not directly quantify intakes of either protein or lipid. Given that the protein leverage hypothesis directly relates to intake, it would be interesting to examine the intake of the diets used here and see if they match the patterns observed for body composition. Future studies should also look to test whether a particular body composition is achieved via protein excretion and whether the costs of excreting protein could be one explanation for the emerging survival cost of being maintained on a high-protein diet (Fanson et al., 2012).

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DATA ACCESSIBILITY

The datasets and materials analysed during the current study are available in the Dryad Repository, doi: 10.5061/dryad.k3rv4.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

JPM and CAW conceived and designed the study. Data collection was carried out by JPM, EH, FM, and AK. Body composition analysis was carried out by CH and JRS at the University of Aberdeen. Statistical analysis was carried out by JPM and CAW. JPM wrote the initial draft of the manuscript, and CAW and JPM performed revisions. All authors approved the final version of the manuscript.

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REFERENCES

Aletor, V., Hamid, I., Niess, E., & Pfeffer, E. (2000). Low-protein amino acid-supplemented diets in broiler chickens: Effects on performance, carcass characteristics, whole-body composition and efficiencies of nutrient utilization. *Journal of the Science of Food and Agriculture*, *80*, 547–554. https://doi.org/10.1002/(ISSN)1097-0010

Alvarez, D., & Metcalfe, N. (2005). Catch-up growth and swimming performance in threespine sticklebacks (*Gasterosteus aculeatus*): Seasonal changes in the cost of compensation. *Canadian Journal of Fisheries and Aquatic Sciences*, *62*, 2169–2176. https://doi.org/10.1139/f05-130

Andrews, R., & Ørskov, E. (1970). The nutrition of the early weaned lamb: I. The influence of protein concentration and feeding level on rate of gain in body weight. *The Journal of Agricultural Science*, *75*, 11–18. https://doi.org/10.1017/S0021859600025995

Barber, I., & Arnott, S. A. (2000). Split-clutch IVF: A technique to examine indirect fitness consequences of mate preferences in sticklebacks. *Behaviour*, *137*, 1129–1140. https://doi.org/10.1163/156853900502484

Barzilai, N., Banerjee, S., Hawkins, M., Chen, W., & Rossetti, L. (1998). Caloric restriction reverses hepatic insulin resistance in aging rats by decreasing visceral fat. *Journal of Clinical Investigation*, *101*, 1353. https://doi.org/10.1172/JCI485

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 48. https://doi.org/10.18637/jss.v067.i01

Bentley, K. T., & Schindler, D. E. (2013). Body condition correlates with instantaneous growth in stream-dwelling rainbow trout and arctic grayling. *Transactions of the American Fisheries Society*, *142*, 747–755. https://doi.org/10.1080/00028487.2013.769899

Boulton, K., Grimmer, A. J., Rosenthal, G. G., Walling, C. A., & Wilson, A. J. (2014). How stable are personalities? A multivariate view of behavioural variation over long and short timescales in the sheepshead swordtail, *Xiphophorus birchmanni*. *Behavioral Ecology and Sociobiology*, *68*, 791–803. https://doi.org/10.1007/s00265-014-1692-0

Carey, J. R., Harshman, L. G., Liedo, P., Muller, H. G., Wang, J. L., & Zhang, Z. (2008). Longevity–fertility trade-offs in the tephritid fruit fly, *Anastrepha ludens*, across dietary-restriction gradients. *Aging Cell*, *7*, 470–477. https://doi.org/10.1111/ace.2008.7.issue-4

Chiba, T., Tamashiro, Y., Park, D., Kusudo, T., Fujie, R., Komatsu, T., … Mori, R. (2014). A key role for neuropeptide Y in lifespan extension and cancer suppression via dietary restriction. *Scientific Reports*, *4*, 4517. https://doi.org/10.1038/srep04517

Colman, R., Roecker, E., Ramsey, J. J., & Kemnitz, J. (1998). The effect of dietary restriction on body composition in adult male and female rhesus macaques. *Aging Clinical and Experimental Research*, *10*, 83–92. https://doi.org/10.1007/BF03339642

De Silva, S. S., Gunasekera, R. M., & Shim, K. (1991). Interactions of varying dietary protein and lipid levels in young red tilapia: Evidence of protein sparing. *Aquaculture*, *95*, 305–318. https://doi.org/10.1016/0044-8486(91)90096-P

Delimaris, I. (2013). Adverse effects associated with protein intake above the recommended dietary allowance for adults. *ISRN Nutrition*, *2013*, 126929. http://doi.org/10.5402/2013/126929

Donaldson, W. E., Combs, G. F., & Romoser, G. L. (1956). Studies on energy levels in poultry rations. The effect of calorie-protein ratio of the ration on growth, nutrient utilization and body composition of chicks. *Poultry Science*, *35*, 1100–1105. https://doi.org/10.3382/ps.0351100

Fanson, B. G., Fanson, K. V., & Taylor, P. W. (2012). Cost of reproduction in the Queensland fruit fly: Y-model versus lethal protein hypothesis. *Proceedings of the Royal Society of London B: Biological Sciences*, *279*, 4893–4900. https://doi.org/10.1098/rspb.2012.2033

Fanson, B. G., Weldon, C. W., Pérez-Staples, D., Simpson, S. J., & Taylor, P. W. (2009). Nutrients, not caloric restriction, extend lifespan in Queensland fruit flies (*Bactrocera tryoni*). *Aging Cell*, *8*, 514–523. https://doi.org/10.1111/ace.2009.8.issue-5

Fontana, L., & Partridge, L. (2015). Promoting health and longevity through diet: From model organisms to humans. *Cell*, *161*, 106–118. https://doi.org/10.1016/j.cell.2015.02.020

Guillaume, J. (2001). *Nutrition and feeding of fish and crustaceans*. Berlin, Germany: Springer Science & Business Media.

Hadfield, J. D. (2010). MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R package. *Journal of Statistical Software*, *33*, 1–22. https://doi.org/10.18637/jss.v033.i02

Hariri, N., & Thibault, L. (2010). High-fat diet-induced obesity in animal models. *Nutrition Research Reviews*, *23*, 270–299. https://doi.org/10.1017/S0954422410000168

Heaney, R. P. (1998). Excess dietary protein may not adversely affect bone. *The Journal of Nutrition*, *128*, 1054–1057.

Helland, S., & Grisdale-Helland, B. (1998). Growth, feed utilization and body composition of juvenile Atlantic halibut (*Hippoglossus hippoglossus*) fed diets differing in the ratio between the macronutrients. *Aquaculture*, *166*, 49–56. https://doi.org/10.1016/S0044-8486(98)00273-7

Hempenstall, S., Picchio, L., Mitchell, S. E., Speakman, J. R., & Selman, C. (2010). The impact of acute caloric restriction on the metabolic phenotype in male C57BL/6 and DBA/2 mice. *Mechanisms of Ageing and Development*, *131*, 111–118. https://doi.org/10.1016/j.mad.2009.12.008

Huang, X., Hancock, D. P., Gosby, A. K., McMahon, A. C., Solon, S., Le Couteur, D. G., … Simpson, S. J. (2013). Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice. *Obesity*, *21*, 85–92. https://doi.org/10.1002/oby.20007

Jensen, K., McClure, C., Priest, N. K., & Hunt, J. (2015). Sex-specific effects of protein and carbohydrate intake on reproduction but not lifespan in *Drosophila melanogaster*. *Aging Cell*, *14*, 605–615. https://doi.org/10.1111/acel.2015.14.issue-4

Kim, J., & Kaushik, S. (1992). Contribution of digestible energy from carbohydrates and estimation of protein/energy requirements for growth of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *106*, 161–169. https://doi.org/10.1016/0044-8486(92)90200-5

Kitano, J., Mori, S., & Peichel, C. L. (2007). Sexual dimorphism in the external morphology of the threespine stickleback (*Gasterosteus aculeatus*). *Copeia*, *2007*, 336–349. https://doi.org/10.1643/0045-8511(2007)7[336:SDITEM]2.0.CO;2

Lee, K. P. (2015). Dietary protein: Carbohydrate balance is a critical modulator of lifespan and reproduction in *Drosophila melanogaster*: A test using a chemically defined diet. *Journal of Insect Physiology*, *75*, 12–19. https://doi.org/10.1016/j.jinsphys.2015.02.007

Lee, W.-S., Monaghan, P., & Metcalfe, N. B. (2013). Experimental demonstration of the growth rate–lifespan trade-off. *Proceedings of the Royal Society B: Biological Sciences*, *280*, 20122370. https://doi.org/10.1098/rspb.2012.2370

Lee, K. P., Simpson, S. J., Clissold, F. J., Brooks, R., Ballard, J. W., Taylor, P. W., … Raubenheimer, D. (2008). Lifespan and reproduction in *Drosophila*: New insights from nutritional geometry. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 2498–2503. https://doi.org/10.1073/pnas.0710787105

Liao, C.-Y., Rikke, B. A., Johnson, T. E., Gelfond, J. A. L., Diaz, V., & Nelson, J. F. (2011). Fat Maintenance is a predictor of the murine lifespan response to dietary restriction. *Aging Cell*, *10*, 629–639. https://doi.org/10.1111/acel.2011.10.issue-4

Maklakov, A. A., Simpson, S. J., Zajitschek, F., Hall, M. D., Dessmann, J., Clissold, F., … Brooks, R. C. (2008). Sex-specific fitness effects of nutrient intake on reproduction and lifespan. *Current Biology*, *18*, 1062–1066. https://doi.org/10.1016/j.cub.2008.06.059

McCay, C. M., Crowell, M. F., & Maynard, L. A. (1935). The effect of retarded growth upon the length of life span and upon the ultimate body size. *The Journal of Nutrition*, *10*, 63–79.

Mitchell, S. E., Delville, C., Konstantopedos, P., Derous, D., Green, C. L., Wang, Y., … Chen, L. (2016). The effects of graded levels of calorie restriction: V. Impact of short term calorie and protein restriction on physical activity in the C57BL/6 mouse. *Oncotarget*, *7*, 19147–19170. https://doi.org/10.18632/oncotarget.v7i15

Mitchell, S. E., Tang, Z., Kerbois, C., Delville, C., Konstantopedos, P., Bruel, A., … Goodyear, S. R. (2015). The effects of graded levels of calorie restriction: I. impact of short term calorie and protein restriction on body composition in the C57BL/6 mouse. *Oncotarget*, *6*, 15902–15930. https://doi.org/10.18632/oncotarget.v6i18

Moatt, J. P., Nakagawa, S., Lagisz, M., & Walling, C. A. (2016). The effect of dietary restriction on reproduction: A meta-analytic perspective. *BMC Evolutionary Biology*, *16*, 1–9. https://doi.org/10.1186/s12862-016-0768-z

Muzumdar, R., Allison, D. B., Huffman, D. M., Ma, X., Atzmon, G., Einstein, F. H., … Barzilai, N. (2008). Visceral adipose tissue modulates mammalian longevity. *Aging Cell*, *7*, 438–440. https://doi.org/10.1111/j.1474-9726.2008.00391.x

Nakagawa, S., Lagisz, M., Hector, K. L., & Spencer, H. G. (2012). Comparative and meta-analytic insights into life extension via dietary restriction. *Aging Cell*, *11*, 401–409. https://doi.org/10.1111/j.1474-9726.2012.00798.x

Park, S., Komatsu, T., Kim, S. E., Tanaka, K., Hayashi, H., Mori, R., & Shimokawa, I. (2017). Neuropeptide Y resists excess loss of fat by lipolysis in calorie-restricted mice: A trait potential for the life-extending effect of calorie restriction. *Aging Cell*, *16*, 339–348. https://doi.org/10.1111/acel.2017.16.issue-2

Partridge, L., Gems, D., & Withers, D. J. (2005). Sex and death: What is the connection? *Cell*, *120*, 461–472. https://doi.org/10.1016/j.cell.2005.01.026

Picard, F., & Guarente, L. (2005). Molecular links between aging and adipose tissue. *International Journal of Obesity and Related Metabolic Disorders*, *29*, S36–S39. https://doi.org/10.1038/sj.ijo.0802912

Ponton, F., Wilson, K., Holmes, A., Raubenheimer, D., Robinson, K. L., & Simpson, S. J. (2015). Macronutrients mediate the functional relationship between *Drosophila* and *Wolbachia*. *Proceedings of the Royal Society of London B: Biological Sciences*, *282*, 20142029.

R Core Team (2016). *R: A language and environment for statistical computing*. Vienna, Austria. http://www.R-project.org/.

Ruohonen, K., Koskela, J., Vielma, J., & Kettunen, J. (2003). Optimal diet composition for European whitefish (*Coregonus lavaretus*): Analysis of growth and nutrient utilisation in mixture model trials. *Aquaculture*, *225*, 27–39. https://doi.org/10.1016/S0044-8486(03)00275-8

Ruohonen, K., Simpson, S. J., & Raubenheimer, D. (2007). A new approach to diet optimisation: A re-analysis using European whitefish (*Coregonus lavaretus*). *Aquaculture*, *267*, 147–156. https://doi.org/10.1016/j.aquaculture.2007.02.051

Selman, C. (2014). Dietary restriction and the pursuit of effective mimetics. *Proceedings of the Nutrition Society*, *73*, 260–270. https://doi.org/10.1017/S0029665113003832

Selman, C., Phillips, T., Staib, J. L., Duncan, J. S., Leeuwenburgh, C., & Speakman, J. R. (2005). Energy expenditure of calorically restricted rats is higher than predicted from their altered body composition. *Mechanisms of Ageing and Development*, *126*, 783–793. https://doi.org/10.1016/j.mad.2005.02.004

Shanley, D. P., & Kirkwood, T. B. L. (2000). Calorie restriction and aging: A life-history analysis. *Evolution*, *54*, 740–750. https://doi.org/10.1111/evo.2000.54.issue-3

Simpson, S. J., Le Couteur, D. G., Raubenheimer, D., Solon-Biet, S. M., Cooney, G. J., Cogger, V. C., & Fontana, L. (2017). Dietary protein, aging and nutritional geometry. *Ageing Research Reviews.*, *39*, 78–86. https://doi.org/10.1016/j.arr.2017.03.001

Simpson, S. J., & Raubenheimer, D. (1995). The geometric analysis of feeding and nutrition: A user's guide. *Journal of Insect Physiology*, *41*, 545–553. https://doi.org/10.1016/0022-1910(95)00006-G

Simpson, S., & Raubenheimer, D. (2005). Obesity: The protein leverage hypothesis. *Obesity Reviews*, *6*, 133–142. https://doi.org/10.1111/obr.2005.6.issue-2

Simpson, S. J., & Raubenheimer, D. (2007). Caloric restriction and aging revisited: The need for a geometric analysis of the nutritional bases of aging. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *62*, 707–713. https://doi.org/10.1093/gerona/62.7.707

Simpson, S. J., Sibly, R. M., Lee, K. P., Behmer, S. T., & Raubenheimer, D. (2004). Optimal foraging when regulating intake of multiple nutrients. *Animal Behaviour*, *68*, 1299–1311. https://doi.org/10.1016/j.anbehav.2004.03.003

Skorupa, D. A., Dervisefendic, A., Zwiener, J., & Pletcher, S. D. (2008). Dietary composition specifies consumption, obesity, and lifespan in *Drosophila melanogaster*. *Aging Cell*, *7*, 478–490. https://doi.org/10.1111/ace.2008.7.issue-4

Solon-Biet, S. M., McMahon, A. C., Ballard, J. W. O., Ruohonen, K., Wu, L. E., Cogger, V. C., … Melvin, R. G. (2014). The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in *ad libitum*-fed mice. *Cell Metabolism*, *19*, 418–430. https://doi.org/10.1016/j.cmet.2014.02.009

Solon-Biet, S. M., Walters, K. A., Simanainen, U. K., McMahon, A. C., Ruohonen, K., Ballard, J. W. O., … Simpson, S. J. (2015). Macronutrient balance, reproductive function, and lifespan in aging mice. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 3481–3486. https://doi.org/10.1073/pnas.1422041112

Sørensen, A., Mayntz, D., Raubenheimer, D., & Simpson, S. J. (2008). Protein-leverage in mice: The geometry of macronutrient balancing and consequences for fat deposition. *Obesity*, *16*, 566–571. https://doi.org/10.1038/oby.2007.58

Speakman, J. R., & Mitchell, S. E. (2011). Caloric restriction. *Molecular Aspects of Medicine*, *32*, 159–221. https://doi.org/10.1016/j.mam.2011.07.001

Speakman, J., Mitchell, S. E., & Mazidi, M. (2016). Calories or protein? The effect of dietary restriction on lifespan in rodents is explained by calories alone. *Experimental Gerontology*, *86*, 28–38. https://doi.org/10.1016/j.exger.2016.03.011

Tarnopolsky, M. A., Atkinson, S. A., MacDougall, J. D., Chesley, A., Phillips, S., & Schwarcz, H. P. (1992). Evaluation of protein requirements for trained strength athletes. *Journal of Applied Physiology*, *73*, 1986–1995.

Terzibasi, E., Lefrançois, C., Domenici, P., Hartmann, N., Graf, M., & Cellerino, A. (2009). Effects of dietary restriction on mortality and age-related phenotypes in the short-lived fish Nothobranchius furzeri. *Aging Cell*, *8*, 88–99. https://doi.org/10.1111/ace.2009.8.issue-2

Vergara, J. M., Robainà, L., Izquierdo, M., & De La Higuera, M. (1996). Protein sparing effect of lipids in diets for fingerlings of gilthead sea bream. *Fisheries Science*, *62*, 624–628. https://doi.org/10.2331/fishsci.62.624

Wootton, R. J. (1984). *A functional biology of sticklebacks*. Berkeley, CA: University of California Press. https://doi.org/10.1007/978-1-4615-8513-8

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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